# DENGUE INFECTIONS IN WEST JAVA, INDONESIA: CURRENT SITUATION AND CHALLENGES

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# DENGUE INFECTIONS IN WEST JAVA, INDONESIA: CURRENT SITUATION AND CHALLENGES

**Proefschift**

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen,
op gezag van de Rector Magnificus prof. mr. S.C.J.J. Kortmann,
volgens besluit van het college van decanen
in het openbaar te verdedigen op maandag 2 juni 2014
om 14.00 uur precies

door

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Geboren 10 September 1965 te Bandung, Indonesië

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**Doctoral Thesis**

to obtain the degree of doctor
from Radboud University Nijmegen
on the authority of the Rector Magnificus prof. dr. S.C.J.J. Kortmann,
according to the decision of the Council of Deans
to be defended in public on Monday 2th June 2014
at 2 pm hours

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# DENGUE INFECTIONS IN WEST JAVA, INDONESIA: CURRENT SITUATION AND CHALLENGES

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# Chapter 1

INTRODUCTION

## INTRODUCTION

Fever is a common symptom in Indonesian patients and often a sign of an ongoing infectious disease. Several studies analyzed the etiology of fever in Indonesia over time: the first was conducted by Anderson in 1971-72 in Jakarta [1], followed by Olson in Klaten, Central Java in 1978 [2], Suharti in Semarang in 1995 [3], Tjitra in Sumba in 1998 [4] and Vollaard in Jakarta in 2002-2003 [5]. The latest study was carried out by Gasem in Semarang in 2005 and 2006 [6]. Most studies were designed to determine specific etiologies and only in one study was a thorough search on different etiologies conducted. Consequently, these different studies produced a variety of results and do not provide a comprehensive picture on the prevalence of infectious diseases in Indonesia. According to these studies, dengue was one of the most common etiologies of fever in Indonesia.

Dengue virus is a positive strand RNA virus belonging to the *Flaviviridae* family [7]. Four antigenically different serotypes of dengue virus have been identified: DENV-1, DENV-2, DENV-3 and DENV-4. Dengue virus is transmitted by *Aedes aegypti* and *Aedes albopictus* mosquitoes, and this virus is therefore also grouped into the *arthropod-borne (arbo) viruses* [7]. Infections with dengue virus are in the majority of cases asymptomatic, but may manifest with clinical symptoms as well, ranging from a mild febrile illness (dengue fever/DF) to severe disease (dengue hemorrhagic fever/DHF or dengue shock syndrome/DSS) [8]. Outbreaks of dengue fever are now globally documented, but epidemics of DHF/DSS were largely unknown until after the end of World War II, when outbreaks were noticed in Southeast Asia in 1950s. Currently, it is estimated that 50-100 million dengue infections occur each year among the 2.5 billion people that live in tropical and subtropical countries, causing 2000 deaths annually [9].

In Indonesia, dengue was first diagnosed in 58 patients in Surabaya and Jakarta in 1968. Over time, the incidence rate (IR) increased to 70/100 000 in 2007, and cases have been reported in 33 Indonesian provinces [10]. The number of cases fluctuates over time (the IR was only 27.6/100 000 in 2011 [11]), and peaks occur every four or five years. Overall, the mortality rate has gradually declined from

41.4% in 1968 [10] to 0.91% in 2011, although in several provinces it remains high (2-8%) [11]. Since these statistics are mostly based on hospitalized cases, it can be expected that the true IR would be higher. It is however difficult to estimate the accurate IR as dengue is generally undifferentiated from other febrile illnesses, particularly during the acute phase. In addition, no simple, rapid, cheap and reliable diagnostic tests are available that can be used under field conditions to confirm the etiology of fever, including dengue.

Clinically, severe dengue cases may be differentiated from other infectious diseases: plasma leakage and a bleeding tendency that develops during defervescence are typical clinical manifestations of a dengue virus infection that are rarely caused by other pathogens [12]. Despite extensive studies, the underlying pathogenesis of severe dengue disease remains unclear. Several theories are proposed, including: the virulence of strains or serotypes; the sequence of infecting serotypes in individuals with multiple dengue infections; heterologous neutralizing antibodies from previous infections that may enhance current viral infections (antibody dependent enhancement (ADE)); a cytokine storm affecting the permeability of vascular endothelial cells; and genetic factors [13].

Apart from the pathogenesis, preventive strategies have been extensively studied, including a dengue vaccine. The main challenge of vaccine development is the existence of four antigenically distinct but related serotypes, whereby infection with one serotype will not protect that individual from subsequent infections with other serotypes. On the contrary, it is suspected that secondary dengue infections are associated with more severe dengue because of the ADE phenomenon [13]. So far, the main prevention method remains the limitation of mosquito bites on an individual and/or community level, as the results from the first phase III vaccine trial revealed only part protection [14].

Although various scientific reports have addressed dengue virus infections in Indonesia, many issues still need further clarification. Several studies were therefore carried out between 2000 and 2009, including a prospective cohort study for DF/DHF in Indonesian adults, as well as an intensive community

observation and a hospital study. The results from these studies are presented in this thesis.

The epidemiology, virology and pathophysiology/clinical aspects of dengue infections in a prospective cohort study lasting 79 months that included 4380 adults in Bandung, West Java, is described in **chapter 2**. The lack of an animal model means a prospective cohort study is the most suitable approach to determine various aspects of natural dengue infections. Traditionally, dengue is seen as a disease that mostly occurs in children, and cohort studies therefore often do not focus on adults. However, dengue is now frequently reported in adults, and in Brazil severe illness is mostly noticed in adults [10,15-17]. The study described in **chapter 2** therefore focuses on an adult population. As well-documented previous dengue infections may be determined in such a cohort, this study also provides a population of adults with a well-characterized dengue immune status which may be precious for vaccine studies. While **chapter 2** provides data on natural dengue infections in an endemic setting, **chapter 3** reports epidemiological, clinical and virological data obtained from ten large hospitals within the capital city of Jakarta during a dengue outbreak in 2004. This is one of the periodic large outbreaks of dengue that have emerged in Indonesia since 1968, with 50 000 cases and 603 deaths reported.

West Java is a hyper-endemic region for dengue where all four seroytpes are circulating. According to the ADE theory, repeated infections predispose the populations to more severe disease. In **chapter 4** we analyzed and elaborated the details of the presence of three heterologous sequential dengue infections in an adult cohort, including data on neutralizing antibodies before and during each dengue episode.

The differential diagnosis of dengue is broad, while the laboratory confirmation has its limitations. In addition to neutralizing antibody assays, a hemagglutination inhibition (HI) test is also recommended by the WHO [18] to diagnose and/or distinguish primary from secondary infections. The latter is important as it is associated with dengue pathogenesis of mild or severe illnesses. The HI test is broadly recognized as the conventional method and in **chapter 5**, HI is compared

with IgG ELISA antibody assay, using the plaque reduction neutralization antibody test (PRNT) as the gold standard to evaluate performance in distinguishing primary or secondary infections. Serological dengue assays are prone to cross-reactivity from other *Flaviviruses* and usually need paired specimens, limiting their applicability for daily practise in the field. Nevertheless, gold standards such as virus culture and isolation assays are technically difficult and time consuming. On the other hand, molecular techniques such as RT-PCR are promising as results are provided within several hours. However, molecular tests are generally limited by high costs and high technical requirements and are therefore presently only available in large hospitals, universities or research institutes. The limitations of the current tests challenged scientists to produce a rapid and accurate diagnostic test during acute illness so that early treatment can be provided, and the signal can be given to the family and the community to take the necessary precautions [19]. Currently, the NS1 antigen test is produced to fulfill this need. In **chapter 6**, the diagnostic and predictive values of NS1 antigen assay and factors associated with its performance were evaluated. The importance of developing an accurate dengue diagnostic tool is important as the prevention, control and treatment of dengue may differ from other febrile illnesses. **Chapters 7, 8, 9 and 10** focus on four important pathogens that are often considered in the differential diagnosis of febrile patients in Indonesia. **Chapter 7** reports on chikungunya infections among febrile patients in the adult cohort during a six year observation period. **Chapter 8** reports on Indonesian national influenza surveillance, including in Bandung and five other sites in West Java from 2003-2007. **Chapter 9** discusses a case report of a hantavirus infection that was identified during a hospital-based hantavirus surveillance study, followed by rodent investigations in the index case community as well as in a control population. **Chapter 10** describes the first discovery of West Nile virus in Indonesia from a hospitalized patient with thrombocytopenia, indicating the need for further study to determine the importance of this disease as the cause of unknown etiologies of fever and/or neurological symptoms.

Finally, as a safe and effective vaccine for dengue is not yet available, prevention efforts should increase the awareness and knowledge of the population, as discussed in **chapter 11.** Knowledge about dengue was tested during enrollment in the study, while education was undertaken during participation in the study.

Finally, one and half years after enrollment, knowledge was tested again. This chapter reports pre- and post-test results from the volunteers. Increased understanding of transmission patterns can also add to better prevention strategies and therefore a cluster study was conducted in West Jakarta from 2002 to 2003. In the community of 53 index cases, a cluster of 15 family members and close neighbors were assessed for previous and acute dengue infections and monitored daily for two weeks to anticipate the occurrence of dengue episodes (**chapter 12**). Finally, in **chapter 13** the main findings are summarized and future options for research are discussed.

## References

1. Anderson KE, Joseph SW, Nasution R, Sunoto, Butler T, et al. (1976) Febrile illnesses resulting in hospital admission: a bacteriological and serological study in Jakarta, Indonesia. Am J Trop Med Hyg 25: 116-121.
2. Olson JG, Ksiazek TG, Suhandiman, Triwibowo (1981) Zika virus, a cause of fever in Central Java, Indonesia. Trans R Soc Trop Med Hyg 75: 389-393.
3. Suharti C, van Gorp EC, Dolmans WM, Groen J, Hadisaputro S, et al. (2009) Hanta virus infection during dengue virus infection outbreak in Indonesia. Acta Med Indones 41: 75-80.
4. Tjitra E, Suprianto S, Dyer M, Currie BJ, Anstey NM (1999) Field evaluation of the ICT malaria P.f/P.v immunochromatographic test for detection of Plasmodium falciparum and Plasmodium vivax in patients with a presumptive clinical diagnosis of malaria in eastern Indonesia. J Clin Microbiol 37: 2412-2417.
5. Vollaard AM, Ali S, van Asten HA, Widjaja S, Visser LG, et al. (2004) Risk factors for typhoid and paratyphoid fever in Jakarta, Indonesia. JAMA 291: 2607-2615.
6. Gasem MH, Wagenaar JF, Goris MG, Adi MS, Isbandrio BB, et al. (2009) Murine typhus and leptospirosis as causes of acute undifferentiated fever, Indonesia. Emerg Infect Dis 15: 975-977.
7. Beasley DW, Barrett ADT (2008) The infectious agent. In: Halstead SB, editor. Dengue. London: Imperial College Press. pp. 29-57.
8. Farrar J (2008) Clinical features of dengue. In: Halstead SB, editor. Dengue. London: Imperial College Press. pp. 171-191.
9. Halstead SB (2008) Dengue: overview and history. In: Halstead SB, editor. Dengue. London: Imperial College Press. pp. 1-9.
10. Pusdatin (2010) Buletin jendela epidemiologi. Departemen Kesehatan Republik Indonesia. pp. 1-4.
11. Pusdatin (2012) Profil data kesehatan Indonesia tahun 2011. Departemen Kesehatan Republik Indonesia.
12. Martina BE, Koraka P, Osterhaus AD (2009) Dengue virus pathogenesis: an integrated view. Clin Microbiol Rev 22: 564-581.
13. Halstead SB (2008) Pathogenesis: risk factors prior to infection. In: Halstead SB, editor. Dengue. London: Imperial College Press. pp. 219-244.
14. Sabchareon A, Wallace D, Sirivichayakul C, Limkittikul K, Chanthavanich P, et al. (2012) Protective efficacy of the recombinant, live-attenuated, CYD tetravalent dengue vaccine in Thai schoolchildren: a randomised, controlled phase 2b trial. Lancet 380: 1559-1567.

15. Guilarde AO, Turchi MD, Siqueira JB, Jr., Feres VC, Rocha B, et al. (2008) Dengue and dengue hemorrhagic fever among adults: clinical outcomes related to viremia, serotypes, and antibody response. J Infect Dis 197: 817-824.
16. Cavalcanti LP, Vilar D, Souza-Santos R, Teixeira MG (2011) Change in age pattern of persons with dengue, northeastern Brazil. Emerg Infect Dis 17: 132-134.
17. Tantawichien T (2012) Dengue fever and dengue haemorrhagic fever in adolescents and adults. Paediatr Int Child Health 32 Suppl 1: 22-27.
18. SEARO W (1999) Prevention and control of dengue and dengue hemorrhagic fever. New Delhi: WHO SEARO. 1-134 p.
19. Blacksell SD (2012) Commercial dengue rapid diagnostic tests for point-of-care application: recent evaluations and future needs? J Biomed Biotechnol 2012: 151967.

# Chapter 2

## The epidemiology, virology and clinical findings of dengue infections in a large cohort of Indonesian adults

*To be submitted to AJTMH*

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## ABSTRACT

Dengue has emerged as one of the most important infectious diseases in the last five decades. Evidence indicates the expansion of dengue endemic areas and consequently the exponentially increase of dengue virus infections. Furthermore, the clinical manifestations are now well-recognized, including the serious complications, which may occur in children as well as in adults. Clinical aspects and management of this disease in adults have been reported from outbreak investigations and prospective hospital based studies. Here, we report the results from a prospective cohort study where 4380 adults participated in West Java, Indonesia, from 2000-2004 and 2006-2009. A total of 2167 febrile episodes were documented whereby dengue infections were confirmed in 268 cases (12.4%). This proportion ranged from 7.6-41.8% each year. The incidence rate was 17.3 cases/1,000 person years which is 43 times higher than previously reported national or provincial rate. Asymptomatic infections were 2.6 times more frequent than symptomatic infections. According to WHO 1997 classifications, there were 210 dengue fever cases, 53 dengue hemorrhagic fever (DHF) cases (32 DHF grade I, 20 DHF grade II, one dengue shock syndrome), and five unclassified cases. Evidence for sequential dengue infections was seen in seven subjects. All four dengue serotypes circulated every year with DENV-3 infections were associated with a more severe illness. Asymptomatic infections were associated with DENV-4 infections. Sequence analysis suggested that the envelope of isolates from all serotypes were strictly conserved and clustered in genotypes that are commonly found in Indonesia.


## INTRODUCTION

Dengue is caused by infection with one of the four dengue viruses: dengue virus 1 (DENV-1), DENV-2, DENV-3 and DENV-4 [1]. Infection with any of these viruses may result in asymptomatic infection, dengue fever (DF), or the more severe forms dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). DHF and DSS were recognized in Southeast Asia soon after multiple serotypes began to circulate in the 1950s [2,3]. Since then the burden of dengue has increased rapidly with the number of annual cases worldwide rising from 908 in the 1950s

to 925,896 in the 2000s [4]. The number of dengue-endemic countries has also expanded from nine to over than 110 countries nowadays [4,5]. Cases of DHF and DSS have also been increasingly recognized in other regions including South Asia, Latin America and the Pacific [6-9]. Finally, in recent years, DF and DHF/DSS have been observed to become more common in adults [10-12]. Because of the spreading of the virus and the impact of the infection, dengue is nowadays widely recognized as the most important arboviral infection worldwide.

Many clinical and epidemiological studies on dengue have relied on outbreak investigations and hospital based studies [13-23]. These studies provide a wealth of data regarding clinical manifestations, laboratory parameters, pathology, and management of the disease. However, they also have some limitations. Hospital studies, for instance, mostly represent severe cases and do not cover the wide spectrum of the clinical picture of dengue infections in adults. Furthermore, pre-illness sera to determine well-characterized previous dengue and early illness sera to measure circulating predictors of disease severity are not collected. Therefore, there is a need for prospective population-based studies to complement the hospital-based investigations [24]. This form of study is considered the best method to determine the epidemiology of dengue infections in a given geographical area. To study the epidemiology of dengue in Bandung, West Java, Indonesia, we conducted a prospective study in a cohort of adults from August 2000 to June 2004 and from September 2006 to April 2009. The aims of this study were to:

1. determine the incidence rate of symptomatic and asymptomatic infections;
2. determine temporal distribution of dengue serotypes ;
3. characterize the clinical manifestations of dengue disease in adults and;
4. determine whether there is a correlation between severity of disease, infecting virus serotypes, pre-illness immune status and sequence of infections.

Preliminary results of the first two years were published previously [25]. Here, we report a complete observation of the study, covering the epidemiology, virology, immunology and clinical pictures of dengue infections.

# MATERIALS AND METHODS

## Ethical considerations

The study protocol was reviewed and approved by the Institutional Review Boards at the Naval Medical Research Unit No. 2 and the National Institute of Health Research and Development, Ministry of Health, Indonesia (DoD 30855, KS.02.01.2.1.2181 and N2.2006.0001, KS.02.01.2.1.2776) in compliance with all U.S. Federal Regulations governing the protection of human subjects. Each volunteer read and signed a consent form upon enrolment.

## Study design

The study was conducted in two phases: from August 2000 to June 2004 and from September 2006 to April 2009. The first phase was carried out in factories A and B and the second phase in Factories A and C. A cohort of 2978 adult volunteers was prospectively followed during the first phase and 2726 during the second phase. Among these volunteers, 1324 participated in both phases. Details of the study design and procedures are illustrated in Figure 1 and are also described by Porter et al [25]. Briefly, blood was collected during enrolment and every three to four months thereafter. Volunteers who experienced fever were evaluated at the factory clinics for clinical assessment and blood was collected when indicated by study clinicians or nurses. A complete blood count (CBC) and dengue diagnostic tests as described below, were performed. Patients were advised to be hospitalized if their platelet count was less than 150,000/mm3 or at the discretion of the clinic attending physicians.

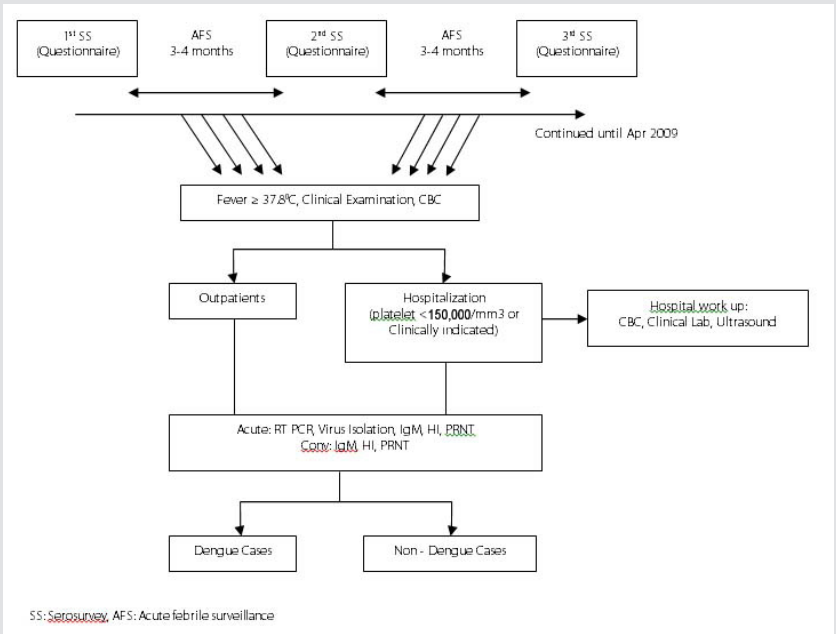


**Figure 1.** Overall study design

## Dengue diagnostic assays

To diagnose dengue infection, virus isolation and RT-PCR were performed on blood specimens collected during the acute phase of illness. Dengue IgM, IgG antibody ELISA (Focus technology), and hemagglutination inhibition (HI) assays were performed on acute and convalescent specimens. A plaque reduction neutralization test (PRNT) was performed on pre-illness, acute and convalescent specimens from confirmed dengue patients. PRNT was also used on paired serosurvey specimens from suspected asymptomatic dengue infection study volunteers. Details of the laboratory procedures have been previously described [25].

## Asymptomatic dengue infections

To estimate the incidence of asymptomatic dengue infections, we randomly selected 675 volunteers, representing 25% of the total volunteer population from September 2006 to February 2008. During this period, serum samples were collected every three to four months from each volunteer up to a total of six serum samples. In order to be able to test thousands of samples for which the HI test is too cumbersome, we developed a new method to identify asymptomatic dengue infections utilizing dengue IgG ELISA (Focus Technology) assays for screening followed by PRNT for confirmation. First, we established an IgG index ratio (IR) that could be used to identify potential asymptomatic infection cases from serial serosurvey samples. In order to do this, we tested 43 paired serosurvey sera, collected before and after confirmed symptomatic dengue episodes, along with 38 paired sera from confirmed non-dengue febrile episodes. A post-/pre-illness IgG index ratio (IR) was calculated in dengue and non-dengue episodes and receiver operating characteristic (ROC) analysis was used to determine the IgG cut-off ratio to identify dengue infections. As samples identified through this screening process were to be further tested by PRNT, a conservative cut-off ratio was chosen in order to ensure that no cases were missed. We chose the lower IR between the lowest IR in the dengue group and the highest IR in non-dengue group as the cut-off value for screening. This resulted in an IR of 1.2. The diagram for these steps is shown in Figure 2A. Upon determining the cut-off IgG IR, six serial serosurvey specimens from 675 volunteers were tested. Specimens with IgG IRs higher than 1.2 between two consecutive serosurvey samples were further tested by PRNT (Figure 2B). The serosurvey samples from each volunteer were tested simultaneously according to the manufacturer's instructions, using the same lot of the kit.

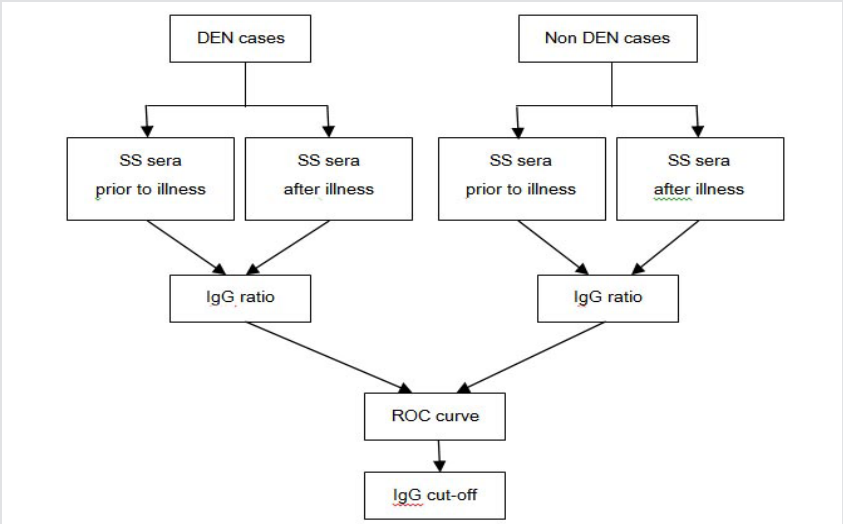


**Figure 2A**. Method to determine the IgG Index Ratio cut-off between post –illness to pre-illness sera

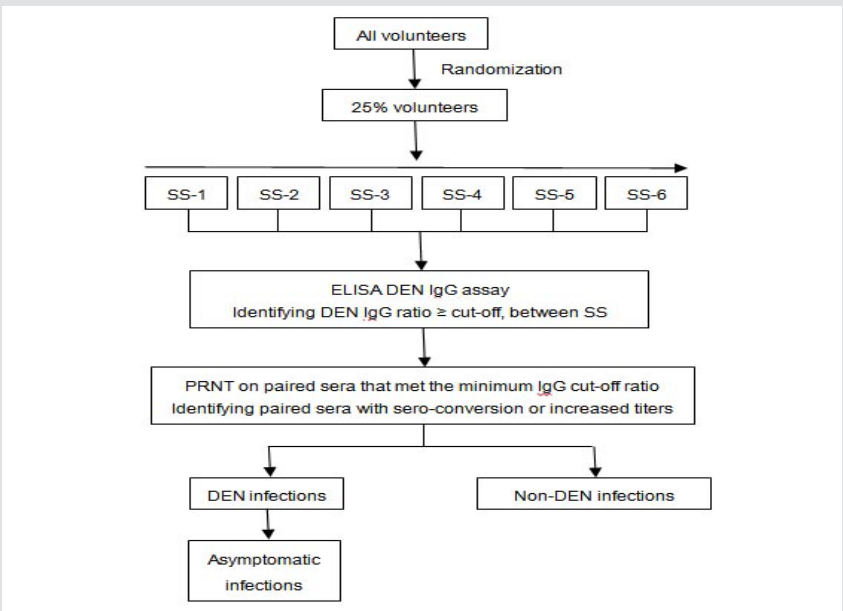


**Figure 2B.** Method for volunteer randomization, screening and confirmation of asymptomatic dengue infections.

## Genotyping/Sequencing analysis

The envelope genes from eight DENV-1, one DENV-2, three DENV-3 and five DENV-4 isolates were sequenced. Viral genome sequencing was conducted based on the methods and primer sets previously described [26-28]. In brief, viral RNA was extracted from virus isolates using Qiamp Viral RNA mini kit (Qiagen, Germany). RNA was used as a template for three RT-PCR assays using different primers set to amplify three overlapping DNA fragments covering the Envelope-NS1 genes (approximately 2700 bases). Specific primer sets were utilized for each serotype. Amplicons were purified using X and the BigDye cycle sequencing kit was used for sequencing reactions (Applied Biosystem, USA) using specific sequencing primers. Sequencing analysis was conducted on a 3130 XL *Genetic Analyzer* (Applied Biosystems) and sequence outputs were assembled using Sequencher software (Genecodes, USA). Phylogenetic trees were generated using the Neighbor Joining method with bootstrapping in MEGA 4 [29]N.I.H.

## Definitions

The following definitions were used in this study:

1. **Dengue infection:** a recent dengue infection was confirmed when DEN virus was isolated, or the RNA was detected in an acute sample, and/or IgM seroconversion, and/or a four-fold or greater increase in HI antibody titers between acute and convalescent specimens was observed.
2. **Primary dengue infection:** a confirmed dengue infection in which dengue IgG antibodies were not detected in the acute sample and an HI titer of ≤ 80 in the convalescent specimen was observed. In cases indeterminate by IgG and HI, cases were also classified as primary infections when $PRNT_{50}$ seroconversion to at least one serotype was detected between acute and convalescent specimens.
3. **Secondary dengue infection:** a confirmed dengue infection in which dengue IgG antibodies or HI antibodies were detected in acute specimens or increased to ≥1280 in convalescent specimens. In cases indeterminate by IgG and HI, cases were classified as secondary infections when the presence

of neutralization antibodies to any serotype in the acute specimens was detected.

4. **Clinical category:** clinical data were analyzed using WHO 1997 criteria. Cases with evidence of plasma leakage but no thrombocytopenia <100,000/mm$^3$ were categorized as unclassified.
5. **Asymptomatic dengue infection:** An asymptomatic dengue infection was confirmed when a four-fold or greater increase of $PRNT_{50}$ titer in any serotype between two serosurvey specimens whose IgG IR ≥ 1.2 was observed.
6. **Naïve population**: a subset of study participants whose serosurvey specimens did not show antibodies to dengue as verified by IgG index <1.
7. **Pre-illness neutralizing Antibody**: pre-illness neutralizing antibodies, as measured by PRNT, were present when the titer >10 and considered protective when the titer was >100 [30].

### Data analysis

Incidence of symptomatic and asymptomatic DEN virus infection was expressed as the number of infections occurring among the cohort per 1,000 person years of follow-up. Volunteers that dropped out from the study were accounted for in the denominator (total person-years) by including only the length of time they were available for follow-up. For comparison between two proportions, the chi-square test was used using STATA 9 software (Texas).

## RESULTS

### Study population

A total of 4380 volunteers were enrolled in the study, 1324 volunteers participated during the entire duration of the study, 1654 only participated in the first phase of the study (August 2000- June 2004) and 1402 only participated in the second phase of the study (September 2006 to April 2009). The mean (SD) age and age range of volunteers at enrolment were 35.6+/-7.7 and 18 to 66 years. A higher proportion of the study population was male (ratio 1.87: 1). Characteristics of the study population aggregated by factory are shown in Table 1.

**Table 1.** The Characteristics of Volunteers

| | Factory A | | | Factory B | Factory C | Total |
|---|---|---|---|---|---|---|
| Duration | Aug 00-June 04 | Aug 06-April 09 | Both periods | Aug 02-June 04 | Aug 06-April 09 | |
| Volunteers:<br>Males<br>Females | <br>451<br>251 | <br>67<br>35 | <br>844<br>480 | <br>438<br>514 | <br>1055<br>245 | <br>2855<br>1525 |
| Age at enrolment<br>Mean (range) | 38.4<br>(18-64) | 35.8<br>(20-53) | 36.2<br>(18-49) | 31.9<br>(18-66) | 36.2<br>(19-55) | 35.6<br>(18-66) |

## Epidemiology of dengue infections

### 1. Seroprevalence and asymptomatic dengue infections

The presence of asymptomatic infections was determined in 675 randomly chosen subjects. No evidence of a previous dengue infection was found in 21 (3.1%) of these subjects. Serological evidence of a previous dengue infection was found in 86.3% of subjects aged 18-27 years, in 96.5% aged 28-37 years, in 96.9% aged 38-47 years, and in all subjects above ≥48 years old.

The occurrence of IgG IRs greater than 1.2 between two consecutive serosurvey samples was found in 35 of 675 volunteers. After being re-tested with PRNT, seven were excluded as no four-fold increase in any serotype was observed (of note: all of the excluded cases had low IgG IRs). Three of the 28 asymptomatic cases were primary infections, two were due to DENV-4 and one was due to DENV-1. We evaluated pre-illness PRNT titers in 25 secondary infection cases. In four cases, the highest antibody titers, all to DENV-3, were lower than the level of the suggested protective neutralizing titer (≥100) [30]. In nine cases, protective neutralizing titers to one serotype were detected, five to DENV-3, two to DENV-1 and one each to DENV-2 and DENV-4. In eight cases, protective neutralizing titers were detected to two serotypes (six to DENV-1 and 3, one to DENV-1 and 2, and one to DENV-2 and 3. Protective neutralizing titers to DENV-1, 2 and 3 were identified in

four cases. In all the secondary cases, pre-illness neutralizing antibodies to DENV-4 were detected in protective titer in 1 case, low titers in 11 and not detected in 13 (76%) cases. During the same time period, 43 symptomatic dengue infections occurred in the cohort resulting in an asymptomatic to symptomatic dengue ratio of 2.6:1.

## 2. Symptomatic dengue infections

A total number of 2,167 febrile episodes occurred during the course of the study, which encompassed a total of 15,454.5 person months of observation. DENV infections were confirmed in 268 episodes, giving an overall proportion of dengue disease among fever patients of 12.4%. This proportion was less than 10% in 2002, 2004, 2006 to 2007, while the highest proportions were observed in 2000 (41.2%) and 2009 (26.6%). The overall incidence rate in the cohort was 17.3/1,000 person years. The annual incidence rate was the lowest in 2006 with 6.3/1,000 person years and the highest in 2009 with 37.8/1,000 person years (Figure 3A). In general, cases started to increase during the rainy season in January, peaked in the first half of the year and then slowly decreased in the second half (Figure 3B).

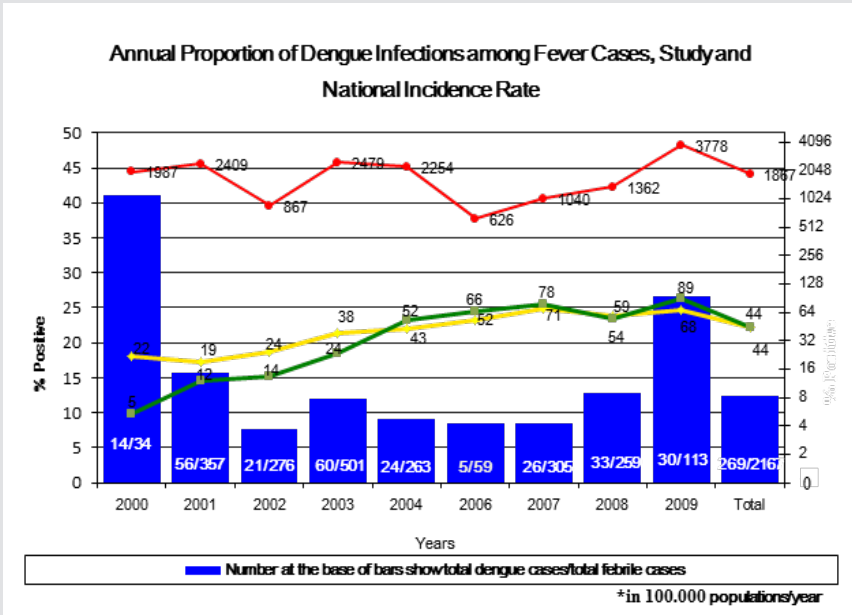


**Figure 3A.** The proportion of dengue infections among febrile episodes by years with the number of cases at the bottom (blue bars), the incidence rate of dengue infections in the population (red), the national incidence rate (yellow) and provincial incidence rate (green)

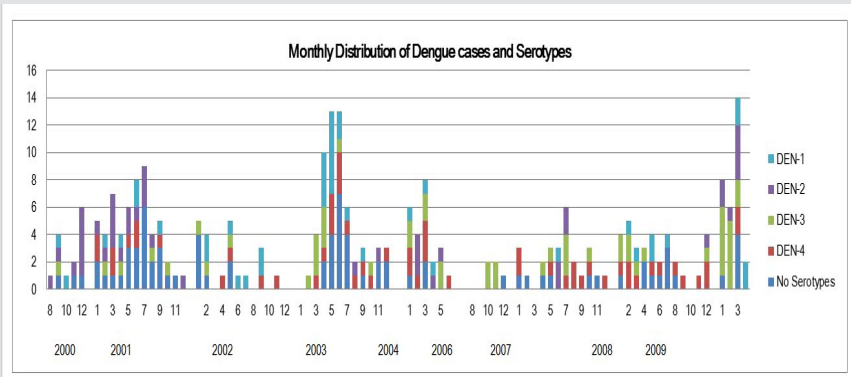


**Figure 3B.** The monthly distribution of dengue cases, break down by serotypes. No surveillance from July 2004 until August 2006.

## Diagnosis and virological findings

Of 268 dengue cases, 92 were confirmed by virus isolation, RT-PCR and serology, 104 by RT-PCR and serology, and 72 only by serological assays. According to HI, IgG ELISA and PRNT antibodies, primary infections were ascertained in 21 (7.8%) and secondary infections in 247 (92.5%) cases. All serotypes were identified in Bandung every year, except DENV-2 which was absent for 18 months from December 2001 until June 2003. DENV-1 and DENV-2 were not detected for nine months from September 2006 to June 2007 and DENV-2 was absent from August 2007 to November 2008. DENV-3 and DENV-4, conversely, were more evenly distributed throughout the year. From a total of 196 cases where the serotype was identified, DENV-4 was the most frequent (28.6%), followed by DENV-3 (26.5%), DENV-2 (22.4%) and DENV-1 (22.4%). The only month that all serotypes were detected to be circulating simultaneously was in March 2009 when concurred with the highest amount of dengue during the study period (Figure 3B).

In confirmed cases, IgM antibodies were positive only in 7.9% (7/89) of subjects who came to the clinics on day two, increased to 20.2% (18/89) on day three, 36.7% (18/49) on day four and 51.3% (20/39) on day five or more. In 16.4% (42/256) of the cases, IgM antibodies were never detected not even on the convalescent specimens. All of these cases were secondary infections.

## Sequencing of envelope genes and genotype analysis

The similarity of envelope gene DENV-1 sequences within isolates from this study and compared to other Indonesian isolates were between 98-99% and 94-98% to 98%, respectively. The similarity of amino acid sequence within this study and other Indonesian isolates was 98-99%, and grouped to genotype IV following classification by Goncalvez 2002. The similarity with Indonesian isolates 2007 (gb/EU448401) was only 97%, resulting in different genotypes. The similarity of envelope gene DENV-2 sequences with Indonesian isolates from 1976 to 2010 was between 97-98% and the similarity of amino acid sequences was 99%. Genotype analysis grouped this isolate into the Cosmopolitan genotype.

Three DENV-3 sequences in the study have similarity of around 96.8-98% with most Indonesian isolates. The similarity in amino acid sequence was 99% and grouped to genotype I. However the sequence similarity with two Indonesian isolates 1998 (AY912454, AY912455) was only 94.5% and the similarity in amino acid sequence was 98%, resulting in a different genotype. Genotype analysis of five DENV-4 isolates placed them in genotype II, together with other Indonesia isolates from 1973 to 2010.

## Clinical categories, signs and symptoms

The majority of cases were DF (78.4%), followed by DHF grade I (11.9%), DHF grade II (7.5%), unclassified (1.9%) and DSS (0.4%). Since patients were advised to come early when they experienced fever, the mean days from fever onset was 3.2 (±1.2) day, ranging from day two to day eight. Symptoms and signs that were frequently reported included myalgia (91.3%), headache (90.9%), arthralgia (63.8%), nausea (59.6%), and a positive tourniquet test (30.9%). Leukopenia (<4000/mm3) and thrombocytopenia (<150,000/mm3) were detected in 12.4% and 17.8% patients, respectively who came to the clinics on day two of illness, 21.3% and 32.6% on day three, 46.2% and 40% on day four and 66.7% and 69.4% on day five or later.

## Sequential dengue infections

Seven patients experienced multiple confirmed dengue febrile illnesses during their participation in this study. Four of them have been reported elsewhere [31]. For the remaining three, two volunteers had evidence of three sequential dengue infections and one had evidence of two dengue infections. The first was a 36 year old male with a probable DENV-3 infection prior to enrolment, followed by unknown serotype, and DENV-1 infections during his participation in the study. The second case was a 33 year old male with evidence of a previous DENV-4 infection, followed by DENV-1 infection and a DENV-3 infection five and half years later. In both cases, the clinical diagnosis was DF. The third case was a 28 year old male with a DENV-2 infection followed by a DENV-3 infection

two years later. Clinically, both episodes manifested as DF with hemorrhagic manifestations that required hospitalization.

## Associations of clinical severity and risk factors

### 1. Clinical severity and serotypes

As described above, 53 (19.8%) cases were classified as the severe form of illness, DHF I, II and DSS. The proportion of DHF/DSS cases among all cases per serotype revealed that DENV-3 tended to be associated with more severe disease (18/52=34.6%), compared to DENV-1 (8/44=18.2%, p=0.07), DENV-2 (8/44=18.2%,p=0.07), and DENV-4 (12/56=21.4%, p=0.12), and to total other serotypes (28/144=19.4%, p=0.03). The significance remained when primary cases were excluded in the analysis. Clinical diagnoses and the infecting serotypes are described in Table 2.

### 2. Clinical outcomes and pre-existing immune status

The severe forms of illness (DHF and DSS) were more frequent in secondary (96.2%) than primary infections (3.8%). The proportion of DHF and DSS among primary cases tended to be lower than secondary cases (9.5% vs 20.6%, p value=0.21). Details of clinical categories and types of infections are summarized in Table 2.

### 3. Clinical outcomes and sequence of infections

There were 27 cases in which the serotype of the second infecting virus was determined by RT-PCR and the serotype of the previous dengue virus infection could be determined through pre-illness neutralizing antibody titers. Previous infections with DENV-1 were identified in five cases, DENV-2 in 19 cases, DENV-3 in one case and DENV-4 in two cases. Following a DENV-2 infection, infections with DENV-3 or DENV-4 resulted in more severe illness than DENV-1 (40%, 28.6%, and 14.3%, respectively). Two DHF cases occurred when DENV-1 was the first infecting virus (2/5), each followed by DENV-2

or DENV-3, and five cases when DENV-2 was the first infecting virus (4/19), two followed by DENV-3, two DENV-4 and one DENV-1. The details were described in Table 3.

**Table 2.** The distribution of clinical severity, type of infections and infecting serotypes

| | Serotypes | | | | |
|---|---|---|---|---|---|
| | **DENV-1 (44)** | **DENV-2 (44)** | **DENV-3 (52)** | **DENV-4 (56)** | **Unknown (72)** |
| Primary infections (21) | 9 | 7 | 4 | 1 | 0 |
| DF(+HM) | 6(1) | 6(1) | 1(2) | 1 | 0 |
| DHF I | 0 | 0 | 0 | 0 | 0 |
| DHF II | 1 | 0 | 1 | 0 | 0 |
| Unclassified | 1 | 0 | 0 | 0 | 0 |
| Secondary infections (247) | 35 | 37 | 48 | 55 | 72 |
| DF(+HM) | 25(1) | 27(2) | 26(5) | 39(3) | 59(5) |
| DHF I | 5 | 5 | 10 | 8 | 4 |
| DHF II | 2 | 3 | 7 | 4 | 2 |
| DSS | 0 | 0 | 0 | 0 | 1 |
| Unclassified | 2 | 0 | 0 | 1 | 1 |

**Table 3.** Clinical severity in cases with evidence of previous and current infecting serotypes

| First infecting serotype (27) | DENV-1 (5) | DENV-2 (19) | DENV-3 (1) | DENV-4 (2) |
|---|---|---|---|---|
| Followed by | DENV-2<br>1 DF, 1 DHF | DENV-1<br>6 DF, 1 DHF | DENV-1 | DENV-1<br>1 DF |
| Followed by | DENV-3<br>1 DHF | DENV-3<br>3 DF, 2 DHF | DENV-2 | DENV-2<br>1 DF |
| Followed by | DENV-4<br>1 DF, 1 DF+HM | DENV-4<br>4 DF, 2 DHF,<br>1 unclassified | DENV-4<br>1 DF | DENV-3 |

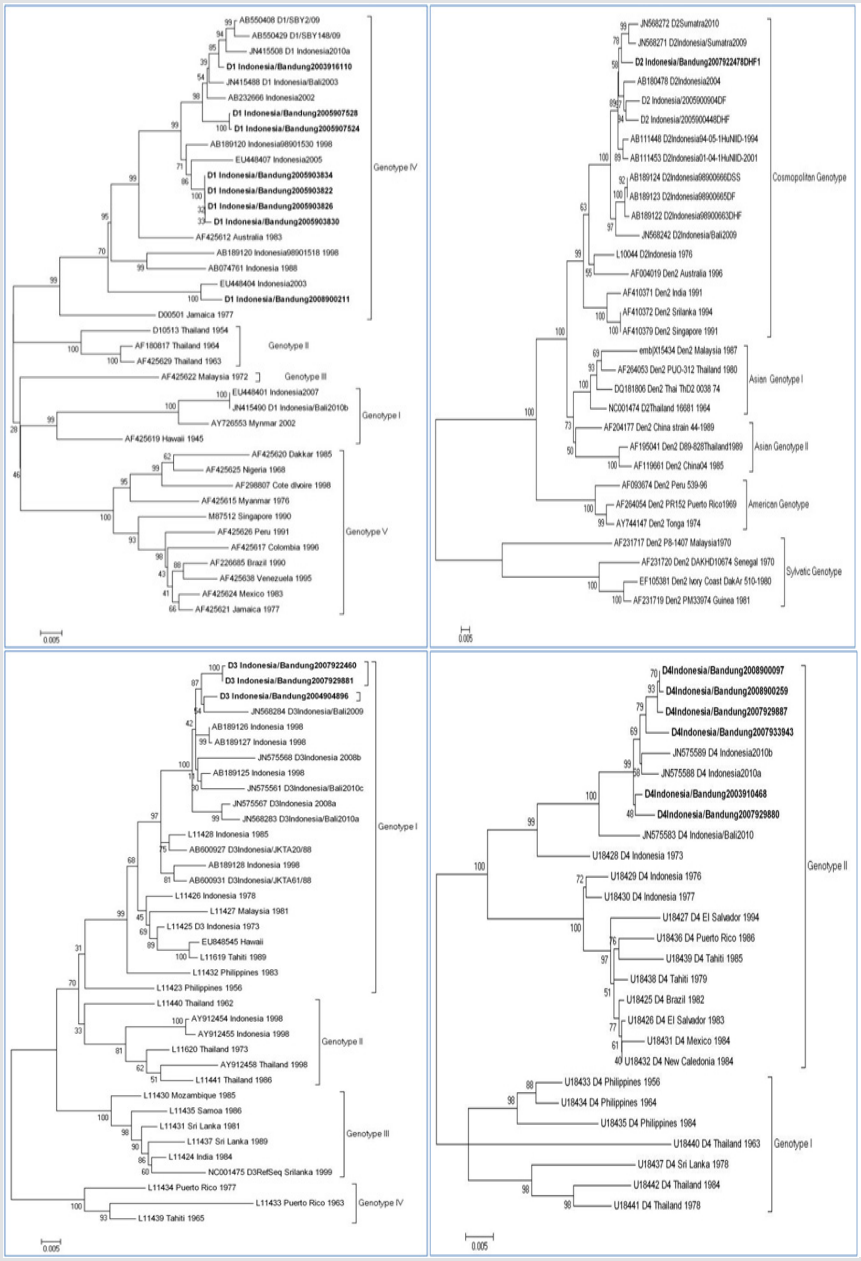


**Figure 4.** Phylogenetic tree of circulating DENV-1 (top left), DENV-2 (top right), DENV-3 (bottom left) and DENV-4 (bottom right)

## DISCUSSION

Our long-term prospective cohort study provides several important findings on the epidemiology of dengue infections in adults living in endemic areas. First, dengue is a major etiology of febrile illness (12.4%) in adults in Bandung, West Java, Indonesia. Second, the average incidence rate from 2000-2004 and 2006-2009 was 43 times higher than the national or provincial rate [32]. Lastly, asymptomatic infections, which provide more accurate information on dengue transmission, were 2.6 times more frequent than symptomatic infections.

The proportion of dengue infections among acute febrile patients in the outpatient setting has rarely been reported in Indonesia. A previous study from 1976, which was a bacteriological and serological survey among febrile patients admitted to hospitals in Jakarta, revealed a similar proportion of dengue infections. [33]. Studies from Malaysia, Myanmar and Thailand report proportions ranging between 5.7 to7%, suggesting dengue is more prevalent in Indonesia [34-36]. The incidence rate in our prospective cohort was calculated from the number of dengue cases that were identified in outpatient clinics, representing both mild and severe cases of dengue illness, whereas previously reported provincial and national incidence rates from Indonesia were based on the hospitalized cases, representing more severe cases. This explains why we found a 43 times higher incidence rate, compared to provincial and national figures.

Furthermore, our study revealed a better estimate of asymptomatic infections, as we monitored a representative proportion of volunteers for one and half years and evaluated the occurrence of dengue infections every quarter of the year. This more detailed information, compared to data derived from yearly serosurvey, gives a better insight into prevalence of dengue and as such transmission rates. The high rate of dengue transmission in this region was in line with our finding that almost all our volunteers have been infected by DENV (96.9%).

Our study revealed that dengue infections in adults were mostly mild (78.4%). This finding is different to previous reports that demonstrate severe cases are more predominant [16,17]. One of the reasons was that our febrile patients came

mostly from outpatients clinics whereas other studies were patients who were indicated to be hospitalized (usually platelet <100,000/mm3). Also, we may underestimate DHF because we relied heavily on serial hematocrit results which may be influenced by fluid therapy, and we used a strict 20% hematocrit increase to confirm hemoconcentration. Although we also performed ultrasonography, at a maximum of once a day it is probably not sufficient as plasma leakage is transient. It was not possible to classify five cases as the clinical manifestations did not fit with any of the categories. All showed plasma leakage but no thrombocytopenia was noticed. Besides the classical signs and symptoms such as fever, headache and myalgia, we found that leukopenia and thrombocytopenia only supported the dengue diagnosis if tested on day five or more of illness. Furthermore, the development of leukopenia and thrombocytopenia concurred with a sensitivity of DENV IgM antibodies above 50%.

The DHF cases were predominantly secondary infections (96.2%). Furthermore, the proportion of DHF cases as part of a secondary infection was higher than in primary infections, but the difference was not significant. In addition, we also identified tertiary infections in six patients, all presenting with DF. This indicates that a previous infection with two dengue serotypes does not protect an individual against future clinical dengue infections.

Our study demonstrated that no serotype was significantly more predominant and almost all serotypes circulated all years in Bandung, except for DENV-1 and DENV-2 for a certain period. The fact that certain serotypes were not detected for some time is probably not caused by the disappearance of these serotypes but may be due to the limited seize of the study population in comparison with the total population of Bandung (approximately 1.5 million). This is supported by the observation that only during the most intensive transmission period (March 2009) all serotypes could be identified. Similar findings were reported during other outbreaks in Indonesia [16,17].

Although any DENV serotypes can cause DHF/DSS, our data revealed that DENV-3 serotype is significantly more frequently associated with severe disease than all other serotypes combined. The higher pathogenicity of DENV-3 was first

discovered in the Philippines and later globally [14,37-41]. Also, in Indonesia, DENV-3 has persistently been reported to cause severe DHF/DSS and to be the predominant serotype in large outbreaks [14,16,17,42,43]. However, the DENV-3 genotypes I and II that are identified in Indonesia [44] are different than genotype III that commonly causes severe disease in other parts of the world. [41]. DENV-3 genotype II, which was identified in Taiwanese citizens returning to Taiwan from Indonesia in 1998, has never been observed since [45].

Apart from serotypes, the sequence of infecting serotypes has also been associated with disease severity [46,47]. We found that most cases with well-characterized sequence serotypes had DENV-2 as the previous infecting serotype. Furthermore DHF more frequently occurred when DENV-3 (40%) was the infecting serotype for the second infection compared to DENV-4 or DENV-1, although this difference was not significant. Our data do not support previous findings from Thailand and Cuba that a DENV-2 infection after DENV-1 is a risk factor to develop a severe dengue [46,47]. The conclusion cannot be drawn regarding other sequences, as the number of cases was too limited.

We suspect that the majority of asymptomatic infections were caused by DENV-4 infections, for the following reasons. First, two of the three primary infections were caused by DENV-4. Second, all pre-illness sera from 24 of 25 secondary asymptomatic infections revealed no or very low neutralizing antibodies to DENV-4. Third DENV-4, along with DENV-3, were the predominant serotypes (39.4% and 42.4%, respectively) that were identified as symptomatic cases during the same period in the same cohort. Asymptomatic or mild form resulting from DENV-4 infections have been reported before from Indonesia [48] and thought to be the reason for the scarcity of DENV-4 hospitalized cases [30].

Finally, our study shows that there are dynamic changes in the various genotypes of the circulating DENV in Indonesia. DENV-1 genotype IV that was detected in our study in 2003, 2005 and 2008 was first detected in 1968 and since then has been endemic in various area in Indonesia [44,45,49]. Regarding other genotypes, DENV-1 genotype I was first reported in Indonesia in 2007 (gb:EU448401) and 2010 in Surabaya [49] and became the predominant genotype in Semarang in

2012, while DENV-1 genotype II has begun to be detected again after the last identification in 1964 in Thailand [50]. Monitoring the dynamic change is very important as it may relate to clinical severity and thus may have public health impact. For example, despite the high homology of DENV-1 isolates from our study with the strain from the 1998 Sumatera outbreak isolates, no significant rise in cases was reported during our study. A plausible explanation based on the genotype analysis was that the 1998 outbreak was caused by the introduction of DENV-1 genotype IV, but from a different clade. Contrasted with the dynamic changes of DENV-1 genotypes, the circulating DENV-2 isolates in Indonesia from 1976 to 2012 that are available in the gene bank, and the isolates from our study, were only grouped into the Cosmopolitan genotype [45,50]. This Cosmopolitan genotype has previously been associated with severe disease [51]. Similarly, the genotype I of DENV-3 in our study is the common genotype in Indonesia and has remained endemic since 1973. However, during the1998 outbreak, the circulating DENV-3 was from genotype II. Our study was one of only a few studies in Indonesia that have successfully detected and isolated DENV-4 [50]. All DENV-4 isolates were similar to the Indonesia isolates from 1973-2010, belonging to genotype II, which were different to the dominant circulating strain in Thailand (genotype I) [26]: and cause mostly mild illness [52].

In conclusion, as our study was a population-based cohort study carried out in large factories in West Java Indonesia with intense monitoring during nearly six years, we were able to identify dengue infections also at an early stage and presenting with minimal symptoms and thus provided accurate epidemiological data regarding the spectrum of dengue disease in adults. In addition, it also provided virological data regarding the serotypes and genotypes circulating and dominating in the region, enable us to make association between clinical severity and pre-illness immune status, infecting serotypes), and/or the sequence of infecting serotypes. Furthermore, this study has identified a population of adults with well-characterized histories of dengue infections, providing preconditions for vaccine-related studies. As dengue continues to be the threat to public health, effective preventive measures have not yet been found and pathogenesis of severe illness is still unclear, further surveillance and studies still need to be conducted.

## ACKNOWLEDGEMENTS

We would like to acknowledge all the volunteers, our research team at Padjadjaran University, Hasan Sadikin Hospital, NAMRU#2 and NIHRD, Ministry of Health, RI, for a long mutual collaboration. In particular, we thank Dr Ardini Raksanagara and Hodijah Satriyo. who were the key leads at the factory, Ratna Irsiana Tan who dedicated most of her time for dengue research, Kendra Chittenden from USAID, Jakarta and INA-RESPOND for their assistance and support during the preparation of this manuscript.

## References


1. Henchal EA, Putnak JR (1990) The dengue viruses. Clin Microbiol Rev 3: 376-396.
2. Kuno G (2009) Emergence of the severe syndrome and mortality associated with dengue and dengue-like illness: historical records (1890 to 1950) and their compatibility with current hypotheses on the shift of disease manifestation. Clin Microbiol Rev 22: 186-201, Table of Contents.
3. Halstead SB, Yamarat C (1965) Recent Epidemics of Hemorrhagic Fever in Thailand. Observations Related to Pathogenesis of a "New" Dengue Disease. Am J Public Health Nations Health 55: 1386-1395.
4. WHO (2009) Dengue guidelines for diagnosis, treatment, prevention and control.
5. Guha-Sapir D, Schimmer B (2005) Dengue fever: new paradigms for a changing epidemiology. Emerg Themes Epidemiol 2: 1.
6. Gupta N, Srivastava S, Jain A, Chaturvedi UC (2012) Dengue in India. Indian J Med Res 136: 373-390.
7. Humayoun MA, Waseem T, Jawa AA, Hashmi MS, Akram J (2010) Multiple dengue serotypes and high frequency of dengue hemorrhagic fever at two tertiary care hospitals in Lahore during the 2008 dengue virus outbreak in Punjab, Pakistan. Int J Infect Dis 14 Suppl 3: e54-59.
8. Tapia-Conyer R, Mendez-Galvan JF, Gallardo-Rincon H (2009) The growing burden of dengue in Latin America. J Clin Virol 46 Suppl 2: S3-6.
9. Bouldouyre MA, Baumann F, Berlioz-Arthaud A, Chungue E, Lacassin F (2006) Factors of severity at admission during an epidemic of dengue 1 in New Caledonia (South Pacific) in 2003. Scand J Infect Dis 38: 675-681.
10. George R, Lam SK (1997) Dengue virus infection--the Malaysian experience. Ann Acad Med Singapore 26: 815-819.
11. Teixeira MG, Costa MC, Coelho G, Barreto ML (2008) Recent shift in age pattern of dengue hemorrhagic fever, Brazil. Emerg Infect Dis 14: 1663.
12. Ibrahim NM, Cheong I (1995) Adult dengue haemorrhagic fever at Kuala Lumpur Hospital: retrospective study of 102 cases. Br J Clin Pract 49: 189-191.
13. Srichaikul T, Punyagupta S, Nitiyanant P, Alkarawong K (1975) Disseminated intravascular coagulation in adult Dengue haemorrhagic fever: Report of three cases. Southeast Asian J Trop Med Public Health 6: 106-114.
14. Sumarmo, Wulur H, Jahja E, Gubler DJ, Suharyono W, et al. (1983) Clinical observations on virologically confirmed fatal dengue infections in Jakarta, Indonesia. Bull World Health Organ 61: 693-701.
15. Gubler DJ, Suharyono W, Lubis I, Eram S, Sulianti Saroso J (1979) Epidemic dengue

hemorrhagic fever in rural Indonesia. I. Virological and epidemiological studies. Am J Trop Med Hyg 28: 701-710.
16. Corwin AL, Larasati RP, Bangs MJ, Wuryadi S, Arjoso S, et al. (2001) Epidemic dengue transmission in southern Sumatra, Indonesia. Trans R Soc Trop Med Hyg 95: 257-265.
17. Suwandono A, Kosasih H, Nurhayati, Kusriastuti R, Harun S, et al. (2006) Four dengue virus serotypes found circulating during an outbreak of dengue fever and dengue haemorrhagic fever in Jakarta, Indonesia, during 2004. Trans R Soc Trop Med Hyg 100: 855-862.
18. Pang J, Salim A, Lee VJ, Hibberd ML, Chia KS, et al. (2012) Diabetes with hypertension as risk factors for adult dengue hemorrhagic fever in a predominantly dengue serotype 2 epidemic: a case control study. PLoS Negl Trop Dis 6: e1641.
19. Leo YS, Thein TL, Fisher DA, Low JG, Oh HM, et al. (2011) Confirmed adult dengue deaths in Singapore: 5-year multi-center retrospective study. BMC Infect Dis 11: 123.
20. Thomas L, Brouste Y, Najioullah F, Hochedez P, Hatchuel Y, et al. (2010) Prospective and descriptive study of adult dengue cases in an emergency department, in Martinique. Med Mal Infect 40: 480-489.
21. Hanafusa S, Chanyasanha C, Sujirarat D, Khuankhunsathid I, Yaguchi A, et al. (2008) Clinical features and differences between child and adult dengue infections in Rayong Province, southeast Thailand. Southeast Asian J Trop Med Public Health 39: 252-259.
22. Chan KP, Lau GK, Doraisingham S, Chan YC (1995) Adult dengue deaths in Singapore. Clin Diagn Virol 4: 213-222.
23. Tripathi BK, Gupta B, Sinha RS, Prasad S, Sharma DK (1998) Experience in adult population in dengue outbreak in Delhi. J Assoc Physicians India 46: 273-276.
24. Endy TP, Yoon IK, Mammen MP (2010) Prospective cohort studies of dengue viral transmission and severity of disease. Curr Top Microbiol Immunol 338: 1-13.
25. Porter KR, Beckett CG, Kosasih H, Tan RI, Alisjahbana B, et al. (2005) Epidemiology of dengue and dengue hemorrhagic fever in a cohort of adults living in Bandung, West Java, Indonesia. Am J Trop Med Hyg 72: 60-66.
26. Klungthong C, Zhang C, Mammen MP, Jr., Ubol S, Holmes EC (2004) The molecular epidemiology of dengue virus serotype 4 in Bangkok, Thailand. Virology 329: 168-179.
27. Zhang C, Mammen MP, Jr., Chinnawirotpisan P, Klungthong C, Rodpradit P, et al. (2005) Clade replacements in dengue virus serotypes 1 and 3 are associated with changing serotype prevalence. J Virol 79: 15123-15130.
28. Zhang C, Mammen MP, Jr., Chinnawirotpisan P, Klungthong C, Rodpradit P, et al.

(2006) Structure and age of genetic diversity of dengue virus type 2 in Thailand. J Gen Virol 87: 873-883.

29. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24: 1596-1599.
30. Endy TP, Nisalak A, Chunsuttitwat S, Vaughn DW, Green S, et al. (2004) Relationship of preexisting dengue virus (DV) neutralizing antibody levels to viremia and severity of disease in a prospective cohort study of DV infection in Thailand. J Infect Dis 189: 990-1000.
31. Kosasih HY, H. Sudjana, P. Alisjahbana, B. Wuryadi, S. Akterono. Tan, RI. Yuwono, D. Beckett, C. Porter, KR. Blair, PJ. (2006) Report of Four Volunteers with Primary, Secondary and Tertiary Dengue Infections during a Prospective Cohort Study. Dengue Bulletin 30: 87-92.
32. Pusdatin (2010) Buletin jendela epidemiologi. Departemen Kesehatan Republik Indonesia. pp. 1-4.
33. Anderson KE, Joseph SW, Nasution R, Sunoto, Butler T, et al. (1976) Febrile illnesses resulting in hospital admission: a bacteriological and serological study in Jakarta, Indonesia. Am J Trop Med Hyg 25: 116-121.
34. Leelarasamee A, Chupaprawan C, Chenchittikul M, Udompanthurat S (2004) Etiologies of acute undifferentiated febrile illness in Thailand. J Med Assoc Thai 87: 464-472.
35. Ellis RD, Fukuda MM, McDaniel P, Welch K, Nisalak A, et al. (2006) Causes of fever in adults on the Thai-Myanmar border. Am J Trop Med Hyg 74: 108-113.
36. Brown GW, Shirai A, Jegathesan M, Burke DS, Twartz JC, et al. (1984) Febrile illness in Malaysia--an analysis of 1,629 hospitalized patients. Am J Trop Med Hyg 33: 311-315.
37. Hammon WM, Rudnick A, Sather GE (1960) Viruses associated with epidemic hemorrhagic fevers of the Philippines and Thailand. Science 131: 1102-1103.
38. Chungue E, Glaziou P, Spiegel A, Martin PM, Roux JF (1992) Estimation of dengue infection attack rate in a cohort of children during a dengue 3 outbreak in Tahiti (1989-1990). Southeast Asian J Trop Med Public Health 23: 157-158.
39. Messer WB, Gubler DJ, Harris E, Sivananthan K, de Silva AM (2003) Emergence and global spread of a dengue serotype 3, subtype III virus. Emerg Infect Dis 9: 800-809.
40. Kanakaratne N, Wahala WM, Messer WB, Tissera HA, Shahani A, et al. (2009) Severe dengue epidemics in Sri Lanka, 2003-2006. Emerg Infect Dis 15: 192-199.
41. de Araujo JM, Bello G, Romero H, Nogueira RM (2012) Origin and evolution of dengue virus type 3 in Brazil. PLoS Negl Trop Dis 6: e1784.
42. Richards AL, Bagus R, Baso SM, Follows GA, Tan R, et al. (1997) The first reported

outbreak of dengue hemorrhagic fever in Irian Jaya, Indonesia. Am J Trop Med Hyg 57: 49-55.

43. Sukri NC, Laras K, Wandra T, Didi S, Larasati RP, et al. (2003) Transmission of epidemic dengue hemorrhagic fever in easternmost Indonesia. Am J Trop Med Hyg 68: 529-535.
44. Raekiansyah M, Pramesyanti A, Bela B, Kosasih H, Ma'roef CN, et al. (2005) Genetic variations and relationship among dengue virus type 3 strains isolated from patients with mild or severe form of dengue disease in Indonesia and Thailand. Southeast Asian J Trop Med Public Health 36: 1187-1197.
45. Ong SH, Yip JT, Chen YL, Liu W, Harun S, et al. (2008) Periodic re-emergence of endemic strains with strong epidemic potential-a proposed explanation for the 2004 Indonesian dengue epidemic. Infect Genet Evol 8: 191-204.
46. Sangkawibha N, Rojanasuphot S, Ahandrik S, Viriyapongse S, Jatanasen S, et al. (1984) Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreak. Am J Epidemiol 120: 653-669.
47. Guzman MG, Deubel V, Pelegrino JL, Rosario D, Marrero M, et al. (1995) Partial nucleotide and amino acid sequences of the envelope and the envelope/nonstructural protein-1 gene junction of four dengue-2 virus strains isolated during the 1981 Cuban epidemic. Am J Trop Med Hyg 52: 241-246.
48. Beckett CG, Kosasih H, Faisal I, Nurhayati, Tan R, et al. (2005) Early detection of dengue infections using cluster sampling around index cases. Am J Trop Med Hyg 72: 777-782.
49. Sjatha F, Takizawa Y, Yamanaka A, Konishi E (2012) Phylogenetic analysis of dengue virus types 1 and 3 isolated in Jakarta, Indonesia in 1988. Infect Genet Evol 12: 1938-1943.
50. Fahri S, Yohan B, Trimarsanto H, Sayono S, Hadisaputro S, et al. (2013) Molecular surveillance of dengue in Semarang, Indonesia revealed the circulation of an old genotype of dengue virus serotype-1. PLoS Negl Trop Dis 7: e2354.
51. Leitmeyer KC, Vaughn DW, Watts DM, Salas R, Villalobos I, et al. (1999) Dengue virus structural differences that correlate with pathogenesis. J Virol 73: 4738-4747.
52. Nisalak A, Endy TP, Nimmannitya S, Kalayanarooj S, Thisayakorn U, et al. (2003) Serotype-specific dengue virus circulation and dengue disease in Bangkok, Thailand from 1973 to 1999. Am J Trop Med Hyg 68: 191-202.

# Chapter 3

## Four dengue virus serotypes found circulating during an outbreak of dengue fever and dengue haemorrhagic fever in Jakarta, Indonesia, during 2004

*Transactions of the Royal Society of Tropical Medicine and Hygiene, 2006*

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## SUMMARY

Periodic outbreaks of dengue have emerged in Indonesia since 1968, with the severity of resulting disease increasing in subsequent years. In early 2004, a purported dengue outbreak erupted across the archipelago, with over 50,000 cases and 603 deaths reported. To confirm the disease aetiology and to provide an epidemiological framework of this epidemic, an investigation was conducted in ten hospitals within the capital city of Jakarta. Clinical and laboratory findings were determined from a cohort of 272 hospitalized patients. Exposure to dengue virus was determined in 180 (66.2%) patients. When clinically assessed, 100 (55.6%) of the 180 patients were classified as having dengue fever (DF), 31 (17.2%) as DF with haemorrhagic manifestations and 49 (27.2%) as dengue haemorrhagic fever (DHF). Evidence from haemagglutination inhibition assays suggested that 33/40 (82.5%) of those with DHF from which laboratory evidence was available suffered from a secondary dengue infection. All four dengue viruses were identified upon viral isolation, with DEN-3 being the most predominant serotype recovered, followed by DEN-4, DEN-2 and DEN-1. In summary, the 2004 outbreak of dengue in Jakarta, Indonesia, was characterized by the circulation of multiple virus serotypes and resulted in a relatively high percentage of a representative population of hospitalized patients developing DHF.

## INTRODUCTION

The dengue virus occurs as four antigenically distinct serotypes (DEN-1, -2, -3 and -4) that have emerged or re-emerged throughout the world since the 17th century [1]. Infection from one or more dengue virus serotypes continues to increase in tropical and subtropical regions, spurred on by an explosion of population growth, urbanization and the spread of the primary vector of disease, the mosquito *Aedes aegypti*. Now recognized in over 100 countries, these viruses are the causative agents of dengue disease and are a threat to almost 40% (2.5 billion) of the world's population. Whilst the majority of dengue infections are asymptomatic, or cause a moderate-to-mild self-limiting febrile illness (dengue fever (DF), sequential heterotypic infections can lead to dengue haemorrhagic fever (DHF) and the more severe dengue shock syndrome (DSS). Infection with

one dengue serotype confers lifelong immunity to that virus. However, previous exposure to a single virus does not provide immunity against subsequent infection with other serotypes. Those living in dengue-endemic areas are often infected with two or more serotypes and are therefore prone to developing severe disease.

Modern times have seen a resurgence of dengue disease in Asia. The first recognized outbreaks of DHF occurred in this region during the 1950s [2,3]. Initial cases in Indonesia were reported in the cities of Surabaya and Jakarta in 1968 [4]. Since then, outbreaks have emerged both in rural and major urban areas of the island chain [5-8]. Over time, an increase in the number of reported dengue cases has become apparent, with a three-fold rise evident in the city of Palembang, South Sumatra, between January and April 1998 relative to historical records [9]. Notwithstanding, the island of Java, where almost 60% of 226 million Indonesians live, has been the region most severely afflicted by periodic outbreaks.

In early 2004, an outbreak of dengue began to spread throughout Indonesia. On 16 February 2004, the Indonesian Ministry of Health declared a national DF/DHF epidemic. Jakarta, the capital city with approximately 16 million inhabitants, was the most affected area [10]. To confirm the aetiology of the outbreak and to define the clinical presentation of the afflicted subjects, the Directorate General, Centers of Disease Control and National Institute of Health Research and Development of the Indonesian Ministry of Health, and the U.S. Naval Medical Research Unit #2 (NAMRU-2) collaborated to conduct an investigation in ten hospitals (Figure 1) across Jakarta from 26 February to 28 March 2004. Results from this study confirmed the circulation of all four dengue virus serotypes and a high incidence of DHF among a cohort of 272 hospitalized patients, the majority of these with laboratory evidence (33/40; 82.5%) occurring in patients who had previously been exposed to dengue.


**Figure 1.** Map showing the position of jakarta, indonesia (inlaid), over a city map with the location of ten hospitals surveyed during the outbreak investigation.

## MATERIALS AND METHODS

### STUDY SITES

Located on the western end of the island of Java, Jakarta is the capital of Indonesia with a population of 16 million people living in a temperate zone with little seasonal variation in weather and mean temperatures ranging between $23^0$C and $33^0$C. The average annual rainfall in Jakarta is 2000mm$^3$ with a peak falling between December and February. The province of Jakarta has five municipalities: West, North, Central, South and East Jakarta. Each has a similar population (1.3-2.8 million) with a density of approximately 5000 inhabitants/

km$^2$. During the conduct of this investigation, two major hospitals from each municipality were chosen for participation: Sumber Waras and Cengkareng from West Jakarta; Infectious Disease Sulianti Saroso and Kodja from North Jakarta; Tarakan and St Carolus from Central Jakarta; Fatmawati and Tebet from South Jakarta; and Budi Asih and Persahabatan from East Jakarta.

## SURVEILLANCE OF FEBRILE PATIENTS

Patients who presented with an undifferentiated febrile illness denoted by abrupt fever of 2-7 days in the absence of an identifiable focus of infection were surveyed. Enrollees were recruited from within all regions of the city and encompassed a wide socioeconomic range. This study was characterized as part of an outbreak investigation and, therefore, whilst studied by the Institutional Review Board (IRB) of both primary institutes, was declared exempt from formal IRB submission and review.

During an initial visit, clinical histories were gathered and physical examinations were performed on each patient. Signs and symptoms were recorded using a standardized clinical form developed by the Ministry of Health team conducting the investigation. Blood was drawn upon presentation and again just prior to discharge. Results from patients with incomplete records were not included in this review.

## HAEMAGGLUTINATION INHIBITION (HI) ASSAYS

Serum samples were tested at serial two-fold dilutions, between 1:10 and 1:5120 by HI assay as escribed previously [11]. A greater than four-fold rise in HI titre in paired samples, or a titre ≥1280 in an acute sample, was considered evidence of dengue infection [12]. Sera with titres <1280 were tested by ELISA to detect anti-dengue IgM antibodies. Primary dengue infections were determined when acute titres were <10 and convalescent titres were ≤80. Subsequently, secondary infection was determined in samples with acute or convalescent titres ≥1280.

## ANTI-DENGUE ELISA

A commercially available antibody capture ELISA previously optimised within our laboratory was used to test for IgM dengue antibodies [13]. Briefly, 1:100 dilutions of patient sera were tested in 96-microwell plates (Dynatech Laboratories Inc., Chantilly, VA, USA) containing passively adsorbed antigen. Samples were allowed to incubate for 1 hour at $37^0$C, then plates were washed and 100 µl of horseradish peroxidase-conjugated goat anti-human IgM was added per well for 1 hour at $37^0$C. Microwells were washed and paramethylbenzidine/ hydrogen peroxide (TMB/$H_2O_2$) was added. Chromogenic change was indicative of the presence of anti-dengue IgM antibodies in the experimental samples. After 30 minutes, absorbance was measured at 450nm by microplate reader (Dynex Laboratories, Inc.). A positive serum sample was determined as one that exceeded the cutoff calculated for each test as specified by the manufacturer.

## VIRUS ISOLATION AND CHARACTERISATION

Isolation of dengue virus was attempted on acute sera from serologically positive individuals. Approximately 0.2 ml of 1:10 diluted patient serum was inoculated onto a confluent monolayer of C6/36 cells and allowed to adsorb at $35^0$C. Cultures were monitored daily for the appearance of cytopathic effects. Upon development of cytopathic effects, or at day 14, cells were scraped from the plates and stained for the presence of virus by standard immunofluorescence using the flavivirus genus reactive monoclonal antibody 4G2. Samples reactive against 4G2 were subtyped with serotype-specific monoclonal antibodies. QIAamp Viral RNA Mini Kits (Qiagen Inc., Valencia, CA, USA) were used to prepare RNA from 140 µl of sera and nested dengue virus reverse transcriptase (RT)-PCR was conducted to confirm the serotype of the isolates [14].

## CLINICAL DIAGNOSIS

The incubation period following dengue exposure varies from 3 to 14 days [15]. DF typically presents with an abrupt fever of 2-7 days and two of the following signs/symptoms, including: retro-orbital pain, headache, myalgias/

arthralgias and haemorrhagic manifestations (i.e. a positive tourniquet test, epistaxis) [16]. An intense erythematous rash occasionally occurs as temperature spikes. Petechiae appear in some patients as fever declines at the conclusion of illness. DF is a rarely fatal, self-limiting disease that afflicts mostly younger children. In contrast, DHF occurs mostly in older children or adults, but has been seen in younger children in countries where multiple viruses are endemic. It is characterized by a sudden onset of fever and an acute phase that lasts 2-6 days. The acute phase of DHF is not remarkable and is often difficult to distinguish from DF or other tropical illnesses. Signs of DHF include those of DF in addition to cerebral oedema, pleural effusion, haemoconcentration, haematuria and thrombocytopenia (platelet count ≤100,000/$mm^3$) [17]. These symptoms are often followed by a sudden deterioration of haemodynamics in the patient. A tourniquet test may suggest increased capillary fragility to attending physicians. During defervescence, plasma leakage can rapidly lead to a haemorrhagic syndrome if not treated promptly. Extensive plasma leakage throughout the body may result in shock and subsequent death.

The 272 patients in the study were considered probable infections when they demonstrated HI titres of ≥1280 on the acute sample or a four-fold titre increase while hospitalized as defined by established WHO criteria [12]. Additionally, primary infections were determined only when acute titres were <10 and convalescent titres were ≤80, and secondary infection was determined when acute or convalescent titres were ≥1280. Serotypes were confirmed by RTPCR or viral isolation. Laboratory-confirmed cases were also classified according to the WHO guidelines. Those cases with haemorrhagic tendencies that could not meet other criteria for DHF (platelet count <100 000/mm3 or plasma leakage) were classified as DF with haemorrhagic tendencies grade I. Patients yielding evidence of a positive tourniquet test, thrombocytopenia and evidence of plasma leakage were grouped as DHF grade I, whilst those cases with spontaneous bleeding manifestations (petechiae, ecchymoses, purpura, mucosal bleeding, haematemesis or melena) were classified as DHF grade II.

## THEORY

Since emerging in Southeast Asia in the 1950s, subsequent epidemics of dengue have infected more people and occurred with greater frequency. With one of the highest populations in the region, the disease burden has been particularly harsh in Indonesia where the first well characterized outbreak occurred in 1973 with 10,189 cases, followed 15 years later by an epidemic that resulted in 47,573 cases. The most severe epidemic to strike the country appeared in 1998 when over 72,133 individuals were infected and 1414 deaths were reported (R. Kusriastuti, unpublished data). The intensity of dengue transmission has been of utmost public health concern on the island of Java since the early 1980s [4]. In 1982, 71% of all Indonesian cases occurred in Java, rising to 84% in 1983 and 91% in 1984 [8]. Whilst the influence of increased prevalence on severe disease can only be determined by the conduct of focused cohort-based studies, it is probable that the spread of dengue viruses across the archipelago and the increase in total population also contribute to increased DHF rates across Indonesia.

## RESULTS

Between January and February 2004, the Indonesian Ministry of Health reported cases of DF in 30 of 32 providences within the archipelago. In the capital city of Jakarta, a total of 20,503 cases were recorded, with an epidemic peak between March and April (Figure 2). To characterize the outbreak better, hospitalized patients were followed in ten hospitals located throughout the municipality. Patient observations coincided with the epidemic curve seen in Jakarta (Figure 2). Acute and secondary sera were collected from a total of 272 febrile patients surveyed both within paediatric (age 1-14 years) and internal medicine (age >14 years) Wards in the five municipalities of Jakarta: West, 103; Central, 24; North, 47; East, 56; and South, 42 (Table 1). The male to female ratio in the cohort was 1.08 and the age ranged from 1 to 72 years, with a mean of 23.4 years. The majority of cases were over 15 years of age, with 37.9% between 15 and 25 years and 37.5% >25 years old. Just fewer than 5% (13 cases; 4.8%) were 3 years of age or younger and 19.9% were between the ages of 4 and 14 years.

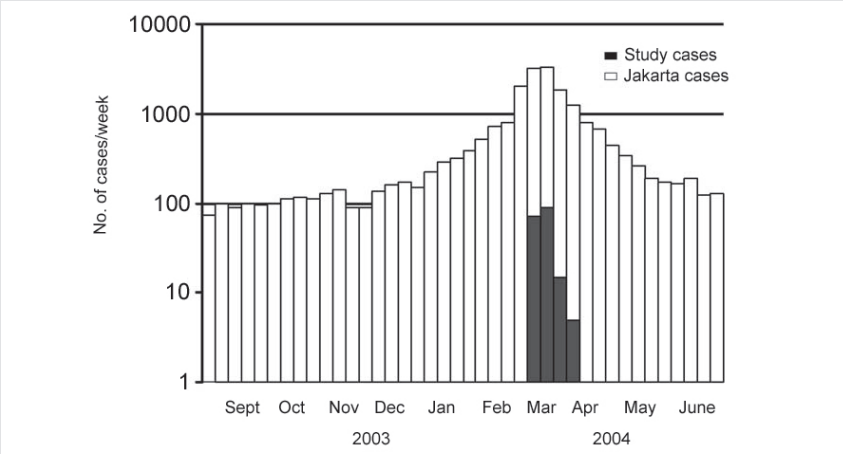


**Figure 2.** Number of reported dengue cases in jakarta (white bars) or cases surveyed (black bars) over an epidemic week. Each bar represents a week. Data span the time September 2003 to june 2004.

**Table 1.** Age and sex of patients per hospital site

| Hospital | No. of patients | 0-3 years | | 4-14 years | | 15-25 years | | >25 years | |
|---|---|---|---|---|---|---|---|---|---|
| | | Male | Female | Male | Female | Male | Female | Male | Female |
| West Jakarta | 103 | | | | | | | | |
| RSSW | 87 | 2 | 5 | 9 | 13 | 6 | 22 | 18 | 12 |
| Cengkareng | 16 | 0 | 0 | 0 | 0 | 6 | 4 | 2 | 4 |
| North Jakarta | 47 | | | | | | | | |
| RSPI | 23 | 2 | 0 | 3 | 4 | 5 | 5 | 3 | 1 |
| Kodja | 24 | 1 | 0 | 7 | 3 | 2 | 6 | 4 | 1 |
| Central Jakarta | 24 | | | | | | | | |
| St. Carolus | 13 | 0 | 0 | 1 | 1 | 3 | 3 | 4 | 1 |
| Tarakan | 11 | 0 | 0 | 0 | 1 | 0 | 5 | 4 | 1 |
| East Jakarta | 56 | | | | | | | | |
| Persahabatan | 36 | 0 | 0 | 1 | 0 | 10 | 6 | 9 | 10 |
| Budi Asih | 20 | 2 | 1 | 2 | 3 | 2 | 1 | 9 | 0 |
| South Jakarta | 42 | | | | | | | | |
| Fatmawati | 37 | 0 | 0 | 1 | 5 | 11 | 5 | 8 | 7 |
| Tebet | 5 | 0 | 0 | 0 | 0 | 0 | 1 | 4 | 0 |
| Total (%) | 272 | 13 (4.8%) | | 54 (19.9%) | | 103 (37.9%) | | 102 (37.5%) | |

RSSW : Sumber Waras; RSPI: Infectious Diseases Sulianti Saroso

Dengue infections were confirmed in 180 (66.2%) of the 272 patients. Of these, 137 (76.1%) were termed probable infection upon a four-fold rise in HI titres of DEN antibodies, 35 (19.4%) by HI titres ≥1280 and 8 (4.4%) by positive anti-dengue IgM (data not shown). When clinically assessed, 100 (55.6%) patients were classified as having DF, 31 (17.2%) as DF with haemorrhagic manifestations (DF + HM) and 49 (27.2%) with DHF. The remaining 92 febrile cases showed no evidence of dengue infection, although those tested who were negative by IgM ELISA (60 cases) when tested prior to day five were deemed inconclusive. Signs, symptoms and common laboratory findings in the reported dengue cases included fever (99.4%), headache (92.2%), thrombocytopenia (78.9%) and nausea (67.2%) (Table 2). Symptoms found more frequently in the group of dengue cases included spontaneous bleeding, haemoconcentration, thrombocytopenia (<100,000/mm$^3$) and leukopenia. Several findings, such as headache, myalgia and positive tourniquet tests, were more common in adults, whereas vomiting, thrombocytopenia and spontaneous bleeding were more common in patients 1-14 years of age.

**Table 2.** Clinical categorization and age among patients

| symptoms | 1-14 years | 15-25 years | >25 years | Confirmed dengue | Non-dengue |
|---|---|---|---|---|---|
| Fever | 43/43 (100%) | 72/72 (100%) | 64/65 (98.5%) | 179/180 (99.4%) | 32/32 (100%) |
| Headache | 38/43 (88.4%) | 66/72 (91.7%) | 62/65 (95.4%) | 166/180 (92.2%) | 23/32 (71.9%) |
| Retro-orbital pain | 9/43 (20.9%) | 21/72 (29.2%) | 20/65 (30.8%) | 50/180 (27.8%) | 6/32 (18.8%) |
| Myalgia | 11/43 (25.6%) | 32/72 (44.4%) | 34/65 (52.3%) | 77/180 (42.8%) | 12/32 (37.5%) |
| Sore throat | 8/43 (18.6%) | 9/72 (12.5%) | 4/65 (6.2%) | 21/180 (11.7%) | 4/32 (12.5%) |
| Nausea | 27/43 (62.8%) | 53/72 (73.6%) | 41/65 (63.1%) | 121/180 (67.2%) | 20/32 (62.5%) |
| Vomiting | 22/43 (51.2%) | 38/72 (52.8%) | 23/65 (35.4%) | 83/180 (46.1%) | 15/32 (46.9%) |
| Abdominal pain | 13/42 (31%) | 29/72 (40.3%) | 22/65 (33.8%) | 64/179 (35.8%) | 8/32 (25%) |
| Positive tourniquet test | 6/16 (37.5%) | 13/25 (52%) | 16/26 (61.5%) | 35/67 (52.2%) | 9/19 (47.4%) |
| Spontaneous haemorrhage [a] | 17/43(39.5%) | 19/72 (26.4%) | 12/65 (18.5%) | 48/180 (26.7%) | 1/32 (3.1%) |
| Haemoconcentration [a] | 12/43 (27.9%) | 28/72 (38.9%) | 18/65 (27.7%) | 58/180 (32.2%) | 1/30 (3.3%) |
| Leukopenia [a] | 19/41 (46.3%) | 40/64 (62.5%) | 27/65 (41.5%) | 86/170 (50.6%) | 4/25 (16%) |
| Thrompocytopenia [a] | 36/43 (83.7%) | 61/72 (84.7%) | 45/65 (69.2%) | 142/180 (78.9%) | 17/32 (53.1%) |

[a] significantly different(p-value <0.05) between dengue and non-dengue cases.

Disease severity was evaluated in 147 of the 180 laboratory-confirmed dengue cases (Table 3). Primary and secondary infections were categorised based on HI titres. Thirty-three patients were excluded from this analysis as results were unclear. Primary infection was identified in 28 (19%) of the cases, whilst secondary infections occurred in 119 (81%) of the 147 confirmed individuals. Sixteen (57.1%) primary infections were characterized as DF, five (17.9%) as DF + HM, whilst seven (25%) were in DHF grades I or II. Of the 119 secondary infections analysed, 64 (53.8%) were categorised as DF, 22 (18.5%) as DF + HM, 25 (21%) as DHF grade I and 8 (6.7%) as DHF grade II. The majority of the primary infections were seen among children 1-14 years old and in (32.1%) younger adults 15-25 years old (46.4%).

Secondary infections occurred predominantly in those aged 15-25 years and >25 years (39.5% and 35.3%, respectively). Overall, a high incidence of DHF (33/40; 82.5%) occurred in patients determined as having a secondary infection. The distribution of DF/DHF cases in those aged 1-14 years was 22 DF, 6 DF +

**Table 3.** Diseases severity and primary versus secondary infection

| Infection type and age | Disease severity | | | |
|---|---|---|---|---|
| | DF | DF+HM | DHF I | DHF II |
| Primary | | | | |
| 1-14 years | 4 | 2 | 3 | 0 |
| 15-25 years | 8 | 2 | 3 | 0 |
| >25 years | 4 | 1 | 0 | 1 |
| Total (%) | 16 (57.1) | 5 (17.9) | 6 (21.4) | 1 (3.6) |
| Combined DHF | | | 7(25) | |
| Secondary | | | | |
| 1-14 years | 18 | 4 | 7 | 1 |
| 15-25 years | 24 | 8 | 10 | 5 |
| >25 years | 22 | 10 | 8 | 2 |
| Total (%) | 64 (53.8) | 22 (18.5) | 25 (21) | 8 (6.7) |
| Combined DHF | | | 33 (27.7) | |
| Overall total (%) [a] | 80 (54.4) | 27 (18.4) | 31 (21.1) | 9 (6.1) |
| Overall DHF | | | 40 (27.2) | |

DF: dengue fever; DF+HM with haemorrhagic manifestations; DHF: dengue haemorrhagic fever.
[a] refers to those in both the primary and secondary category

HM, 10 DHF grade I and 1 DHF grade II. In those patients older than 14 years, the distribution was 58 DF, 21 DF + HM, 21 DHF grade I and 8 DHF grade II. No mortalities occurred among the patients observed.

The infecting dengue serotype was confirmed either by RT-PCR or isolation in 28 acute sera (Table 4). The predominant serotype found in the 2004 outbreak was DEN-3 (18; 64.3%), followed by DEN-4 (4; 14.3%), DEN-2 (4; 14.3%) and DEN-1 (2; 7.1%); there was a single co-infection with DEN-3 and DEN-4. Ten isolates were recovered from 72 serologically confirmed dengue sera, giving an isolation rate of 13.9% overall. DEN-3 was both predominant and widespread, having been found in all five municipalities. Molecular characterization and sequencing of the DEN-3 isolates revealed similarities to previous viruses isolated in Indonesia during a 1998 outbreak (unpublished data).

Disease severity was correlated with the infecting virus in 28 patients from whom the virus was identified (Table 4). Two cases infected with DEN-1 were either DF + HM or DHF grade I, whilst three cases of DEN-2 were DF and one was DHF grade I. Of 18 persons infected with DEN-3, 7 had DF, 4 had DF + HM, 5 had DHF grade I and 2 had DHF grade II. Four cases were infected with DEN-4, which included one DF + HM and three DHF grade I.

**Table 4.** Infecting virus versus disease severity

| Disease severity | Infecting virus | | | | Total |
|---|---|---|---|---|---|
| | DEN-1 | DEN-2 | DEN-3 | DEN-4 | |
| DF (%) [a] | 0 | 3 (30) | 7 (70) | 0 | 10 |
| DF+HM (%) | 1 (17) | 0 | 4 (67) | 1 (17) | 6 |
| DHF grade I (%) [b] | 1 (10) | 1 (10) | 5 (50) | 3 (30) | 10 |
| DHF grade II (%) | 0 | 0 | 2 (100) | 0 | 2 |
| Total (%) [c] | 2 (7.1) | 4 (14.3) | 18 (64.3) | 4 (14.3) | |

DF: dengue fever; DF+HM: DF with haemorrhagic manifestations; DHF; dengue haemorrhagic fever.

[a] Percentage based upon specific disease category.

[b] A single DHF grade I patient was co-infected with both DEN-3 and DEN-4.

[c] Percentage based upon specific dengue serotype.

## DISCUSSION

Beginning in the early months of 2004, Indonesia was stricken by a significant outbreak of dengue. Similar to 1998, this epidemic affected all districts and subdistricts in Jakarta, transitioning urban and rural areas and across socioeconomic boundaries. By its end, tens of thousands of cases of the disease had been recorded in the capital city of Jakarta alone, resulting in 603 deaths [10]. Of note, whilst the magnitude and severity of cases have increased in Indonesia with each subsequent outbreak, over the last decade the case fatality rate has declined as a consequence of the ability of local clinicians to recognise and manage DHF patients.

To confirm disease aetiology and to provide a framework for the 2004 epidemic, an opportunistic hospital-based study was conducted. At the height of the epidemic in Jakarta, evidence for dengue exposure was examined in a cohort of 272 hospitalised patients recruited from ten geographically disparate healthcare centres. Case definition was based upon criteria that included abrupt fever and common signs of dengue infection. An age-based assessment of infection demonstrated that cases were distributed to a greater extent among those 15 years of age or older, whilst it was more limited in younger individuals. This is in contrast to earlier studies in Southeast Asia that showed that dengue infections primarily afflicted paediatric populations, for example a study from Thailand exhibiting a modal age of hospitalised patients of 4-6 years [18]. Indeed, the population often at greatest risk for DHF has been children, although a marked increase in the number of DHF cases in subjects over 15 years old was observed more recently in the Philippines and Malaysia [19]. Follow-up among the patients did not demonstrate a bias towards adults when hospitalisation was considered. Indeed, care has been extended to patients of all ages as, during times of national emergency, the government of Indonesia has provided for the costs incurred by patients.

Clinical symptoms such as fever, headache, nausea, myalgia and vomiting were commonly exhibited by patients seen during the investigation. However, the percentage of patients reporting these symptoms was not significantly different

from those recorded in the non-dengue group. Further, a positive tourniquet test did not discern between those patients with proven dengue and those with a non dengue infection (52.2% and 47.4%, respectively). Signs more frequently correlated with dengue infection within our cohort were spontaneous bleeding, haemoconcentration, leukopenia and thrombocytopenia. These observations are in contrast to a study conducted in Bangladesh that suggested an association between dengue infection and myalgia or vomiting but not thrombocytopenia [20]. Several symptoms, such as headache, retroorbital pain, myalgia and abdominal pain, were less frequently observed in the younger patients than in older age groups. However, thrombocytopenia, spontaneous bleeding and vomiting were more predominantly reported in children.These findings parallel a study conducted in Mumbai in paediatric patients where bleeding manifestations were observed in 54% and thrombocytopenia in 92% of the cohort [21].

Although dengue infections are often asymptomatic, the incidence of DHF increases in parallel with an elevation in heterotypic dengue infections [3,22,23]. As shown in earlier work in Asia [23], our study demonstrated a clear link between morbidity and secondary infection. When clinically assessed, 54.4% of the patients infected with dengue were classified as DF, whilst 45.6% were segregated into either DF + HM or DHF. Evidence from HI suggested that 33/40 (82.5%) of those who developed DHF suffered a secondary infection. Also, the percentage of DHF cases seen in our study during the epidemic was notably higher than that seen in non-outbreak settings on Java. For instance, in a dengue pathogenesis study recruited around an index dengue case, only 1 of 17 dengue infections progressed to DHF, whilst in a disease incidence study among an adult cohort only 5 DHF cases were observed among 90 individuals with documented dengue infection [24].

The pathogenesis of DHF is not well characterised. Among a multitude of contributing factors is the infecting virus serotype. The emergence of a new dengue serotype in a dengue-endemic area often results in an epidemic, as was the case in Myanmar in 2001 [25]. Some data have pointed to an association between dengue serotype, genotypes and disease pathogenesis. For instance,

clinically monotypic infection with the American genotype DEN-2 virus results in an asymptomatic disease. The genetic sequence of the American DEN-2 virus differs from the Asian genotype viruses by approximately 10%. A single change in domain III of E (amino acid 390) is suspected as being associated with decreased virulence of the American virus [26]º. However, little is known about the evolution of virulence among dengue viruses and the pressure of many factors, including passive serotype immunity, likely contribute to disease severity [27].

In this investigation, all four dengue viruses were isolated from patients, although DEN-3 was the predominant serotype. Furthermore, DEN-3 viruses were more prominently associated with DF + HM and DHF. As the DEN-3 viruses in Southeast Asia evolve, it is conceivable that their virulence could be altered. For example, a DEN-3 outbreak in Central Java in 1978 was associated with mild disease and low viraemia [28]. However, recent epidemics attributed to the DEN-3 virus in Asia demonstrate a higher incidence of symptomatic disease [29,30]. Whole genome phylogenic analysis of the DEN-3 virus isolated in our current study (unpublished data) reveals its strong similarity to the DEN-3 strain that emerged and wrecked havoc in 1998. These isolates appear to result in an increase in symptomatic disease, although a correlation between specific base pair changes and viraemia remains circumstantial.

In conclusion, this investigation confirmed the aetiology and provided the epidemiological framework for an outbreak of DF and DHF that emerged in Indonesia in the early months of 2004. Whilst such epidemics have become increasingly more common throughout Southeast Asia in recent times, this one was particularly alarming given the relatively high number of individuals infected and the percentage of adults who developed DHF. Progress in controlling morbidity and mortality from dengue will be gained only when an understanding of the molecular fingerprint of the infecting virus is realised and the confounding role of previous heterotypic infections understood. In the absence of these, public health initiatives involving education and vector control remain at the forefront of dengue control.

## CONFLICTS OF INTEREST STATEMENT

The authors have no conflicts of interest concerning the work reported in this paper.

## ACKNOWLEDGEMENTS

The authors thank Dr Shannon D. Putnam for statistical analysis, Antonius Pradana for technical assistance, and Rudi Hendro Putranto and Eny Muchlas Trianingsih for data collection. We are grateful for the laboratory help provided by Erlin Listiyaningsih, Gustiani, Chairin N. Ma'roef and Sherley Y. Tobing as well as the administrators, nurses and staff at the collaborating hospitals, including: Sumber Waras and Cengkareng from West Jakarta, Infectious Disease Sulianti Saroso and Kodja from North Jakarta, Tarakan and St Carolus from Central Jakarta, Fatmawati and Tebet from South Jakarta, and Budi Asih and Persahabatan from East Jakarta. This investigation was funded jointly by the government of Indonesia in awards to the Director General of Communal Disease Control and Environmental Health (DG-CDC-EH), the NIHRD and by the U.S. Department of Defense's Global Emerging Infection System (GEIS). Views expressed in this article are those of the authors and do not reflect the official policy or position of the Indonesian NIHRD and DG-CDC-EH or the U.S. Department of Defense or Department of the Navy.

## References


1. Gubler DJ (1998) Dengue and dengue hemorrhagic fever. Clin Microbiol Rev 11: 480-496.
2. Halstead SB (1992) The XXth century dengue pandemic: need for surveillance and research. World Health Stat Q 45: 292-298.
3. Halstead SB, Nimmannitya S, Yamarat C, Russell PK (1967) Hemorrhagic fever in Thailand; recent knowledge regarding etiology. Jpn J Med Sci Biol 20 Suppl: 96-103.
4. Nathin MA, Harun SR, Sumarmo (1988) Dengue haemorrhagic fever and Japanese B encephalitis in Indonesia. Southeast Asian J Trop Med Public Health 19: 475-481.
5. Graham RR, Juffrie M, Tan R, Hayes CG, Laksono I, et al. (1999) A prospective seroepidemiologic study on dengue in children four to nine years of age in Yogyakarta, Indonesia I. studies in 1995-1996. Am J Trop Med Hyg 61: 412-419.
6. Gubler DJ, Suharyono W, Lubis I, Eram S, Sulianti Saroso J (1979) Epidemic dengue hemorrhagic fever in rural Indonesia. I. Virological and epidemiological studies. Am J Trop Med Hyg 28: 701-710.
7. Sukri NC, Laras K, Wandra T, Didi S, Larasati RP, et al. (2003) Transmission of epidemic dengue hemorrhagic fever in easternmost Indonesia. Am J Trop Med Hyg 68: 529-535.
8. Sumarmo (1987) Dengue haemorrhagic fever in Indonesia. Southeast Asian J Trop Med Public Health 18: 269-274.
9. Corwin AL, Larasati RP, Bangs MJ, Wuryadi S, Arjoso S, et al. (2001) Epidemic dengue transmission in southern Sumatra, Indonesia. Trans R Soc Trop Med Hyg 95: 257-265.
10. Ahmad A (2004) Dengue death toll rises in Indonesia. Lancet 363: 956.
11. Clarke DH, Casals J (1958) Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Am J Trop Med Hyg 7: 561-573.
12. WHO (1999) Prevention and Control of Dengue and Dengue Haemorrhagic Fever. Comprehensive Guidelines World Health Organization Regional Office for South-East Asia, New Delhi, WHO Regional Publication SEARO No. 29.
13. Porter KR, Widjaja S, Lohita HD, Hadiwijaya SH, Maroef CN, et al. (1999) Evaluation of a commercially available immunoglobulin M capture enzyme-linked immunosorbent assay kit for diagnosing acute dengue infections. Clin Diagn Lab Immunol 6: 741-744.
14. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV (1992) Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase/polymerase chain reaction. J Clin Microbiol 30: 545-551.
15. Halstead SB (1988) Pathogenesis of dengue: challenges to molecular biology. Science 239: 476-481.

16. Waterman SH, Gubler DJ (1989) Dengue fever. Clin Dermatol 7: 117-122.
17. Rothman AL, Ennis FA (1999) Immunopathogenesis of Dengue hemorrhagic fever. Virology 257: 1-6.
18. Nimmannitya S (1987) Dengue haemorrhagic fever in Thailand. Southeast Asian J Trop Med Public Health 18: 291-294.
19. Pinheiro FP, Corber SJ (1997) Global situation of dengue and dengue haemorrhagic fever, and its emergence in the Americas. World Health Stat Q 50: 161-169.
20. Rahman M, Rahman K, Siddque AK, Shoma S, Kamal AH, et al. (2002) First outbreak of dengue hemorrhagic fever, Bangladesh. Emerg Infect Dis 8: 738-740.
21. Shah I, Deshpande GC, Tardeja PN (2004) Outbreak of dengue in Mumbai and predictive markers for dengue shock syndrome. J Trop Pediatr 50: 301-305.
22. Burke DS, Nisalak A, Johnson DE, Scott RM (1988) A prospective study of dengue infections in Bangkok. Am J Trop Med Hyg 38: 172-180.
23. Sangkawibha N, Rojanasuphot S, Ahandrik S, Viriyapongse S, Jatanasen S, et al. (1984) Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreak. Am J Epidemiol 120: 653-669.
24. Porter KR, Beckett CG, Kosasih H, Tan RI, Alisjahbana B, et al. (2005) Epidemiology of dengue and dengue hemorrhagic fever in a cohort of adults living in Bandung, West Java, Indonesia. Am J Trop Med Hyg 72: 60-66.
25. Thu HM, Lowry K, Myint TT, Shwe TN, Han AM, et al. (2004) Myanmar dengue outbreak associated with displacement of serotypes 2, 3, and 4 by dengue 1. Emerg Infect Dis 10: 593-597.
26. Rico-Hesse R, Harrison LM, Salas RA, Tovar D, Nisalak A, et al. (1997) Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. Virology 230: 244-251.
27. Twiddy SS, Holmes EC, Rambaut A (2003) Inferring the rate and time-scale of dengue virus volution. Mol Biol Evol. 20: 122-129.
28. Gubler DJ, Suharyono W, Lubis I, Eram S, Gunarso S (1981) Epidemic dengue 3 in central Java, associated with low viremia in man. Am J Trop Med Hyg 30: 1094-1099.
29. Braga EL, Moura P, Pinto LM, Ignacio SR, Oliveira MJ, et al. (2001) Detection of circulant tumor necrosis factor-alpha, soluble tumor necrosis factor p75 and interferon-gamma in Brazilian patients with dengue fever and dengue hemorrhagic fever. Mem Inst Oswaldo Cruz 96: 229-232.
30. Endy TP, Nisalak A, Chunsuttiwat S, Libraty DH, Green S, et al. (2002) Spatial and temporal circulation of dengue virus serotypes: a prospective study of primary school children in Kamphaeng Phet, Thailand. Am J Epidemiol 156: 52-59.

# Chapter 4

## Report of four volunteers with primary, secondary and tertiary dengue Infections during a prospective cohort study

*Dengue Bulletin – Volume 30, 2006*

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## ABSTRACT

A prospective study of dengue fever (DF) and dengue haemorrhagic fever (DHF) in adults was conducted at two factories in Bandung, Indonesia, between August 2000 and July 2004. A total of 2978 employees were followed for the development of fever and clinical signs of DF/DHF. Among 1431 patients reporting a febrile illness, dengue infections were detected in 177 individuals. Four enrollees with evidence of previous dengue exposure experienced two consecutive episodes of dengue infection. Analysis of pre illness sera revealed that one patient had neutralizing antibodies (nAbs) to DENV-1, two had nAbs to DENV-2, and another had nAbs to DENV-2, 3 and 4. The individual with pre-illness neutralizing antibodies to DENV-1 experienced a DENV-3 secondary infection, followed by a third infection with an unknown serotype. One of the two individuals with pre-illness neutralizing antibodies to DENV-2 experienced a DENV-3 and then a DENV-1 infection. The other DENV-2 immune patient experienced sequential infections with DENV-4 and DENV-3 viruses. Finally, the individual with pre-illness neutralizing antibodies against three dengue viruses had a subsequent infection with DENV-4, followed one year later by DENV-3. In this instance, the patient acquired grade II DHF (WHO criteria) from the subsequent DENV-4 infection. These data provide confirmatory evidence that humans can experience three sequential heterologous dengue infections. Importantly, the occurrence of a second and third infection in individuals with pre-illness antibodies against multiple dengue serotypes indicates that neutralizing antibodies are cross-reactive in vitro but not cross-protective in vivo.

## INTRODUCTION

Dengue fever (DF) and dengue haemorrhagic fever (DHF) are endemic diseases that occur with regularity across Indonesia. The first recognized outbreak of DHF occurred during the 1960s. Since then, outbreaks have occurred both in rural and major urban areas across the archipelago. In recent years, an increase in dengue cases among individuals ≥15 years old had been reported [1,2] but the clinical manifestations of dengue disease progression are poorly understood in adults. To further evaluate the epidemiology of DF and DHF in an adult population, a

prospective dengue cohort study was initiated in adult workers at two textile factories in Bandung, West Java, Indonesia [3]. During the course of this study, enrollees were actively followed up for the development of the disease over a four-year study period.

## METHODS

After obtaining informed consent, 2978 workers were enrolled and medical history questionnaires and demographic data forms completed. The enrollees underwent physical examination and their blood samples were taken. Baseline neutralizing antibody titers to assess their previous dengue exposure in addition to routine laboratory blood counts and chemistries were measured. When a volunteer experienced a febrile illness, a medical evaluation was conducted and blood samples were taken for complete blood counts (CBC), DENV isolation, reverse-transcriptase polymerase chain reaction (RT-PCR) and serology (anti-DENV IgM, haemagglutinationinhibition/HI, and neutralizing antibodies). Volunteers with thrombocoytopenia (platelet count ≤100 000/ $mm^3$) or those appraised as severely ill, were hospitalized. During their hospital stay, haematocrit and platelet counts were evaluated daily, while chest and abdominal sonograms were performed to detect plasma leakage. Convalescent sera were collected from hospitalized and nonhospitalized patients on day 10 through day 14. Serosurveys were conducted every three months to obtain peripheral blood mononuclear cells (PBMCs) and plasma for the evaluation of dengue immune status. Fifty per cent plaque reduction neutralizing antibody titers were determined by probit analysis. Titers below a dilution of 1:20 on a PRNT were considered negative.

An enrollee was considered to have a DENV infection if he or she had a compatible clinical illness and laboratory evidence of DENV, which included either identification of the infecting virus by RT-PCR or virus isolation, the presence of IgM antibodies, or a four-fold increase in HI antibody titer. World Health Organization (WHO) criteria were used to categorize disease status [4].

## RESULTS AND DISCUSSION

From August 2000 until July 2004, 1431 febrile illnesses were evaluated, which resulted in the confirmation of 177 acute dengue infections. Among these, four dengue-immune enrollees experienced two separate (consecutive) episodes of dengue infection.

The first instance of multiple dengue infection occurred in a 42-year-old male who presented to the hospital with fever, headache, myalgia, coryza, nausea and vomiting (Table 1). Analysis of pre-illness serum obtained 2 months prior to his second dengue infection revealed the presence of neutralizing antibodies to DENV-1 ($PRNT_{50}$ titer: 187). The neutralizing antibody profile of the subsequent convalescent serum indicated that the infecting serotype was DENV-3. Five months later, the study volunteer demonstrated anti-DENV IgM antibodies and a four-fold increase in HI antibody titers suggesting a third dengue infection (Table 2). However, in this instance, the infecting serotype was not determined. While both episodes required hospitalization, the first episode was slightly more severe with the onset of thrombocytopenia. Clinically, both episodes were diagnosed as DF.

The second example of consecutive dengue infection was observed in a 29-year old female. In this instance, analysis of neutralizing antibody activity two months prior to the second infection revealed a PRNT50 titer of 359 against DENV-2. The infecting serotype was later confirmed as DENV-3 by RT-PCR. Eighteen months after the second infection, a third infection with DENV-1 was suspected based on the development of nAbs. Only the second DENV-3 infection resulted in the hospitalization of the subject. Non-specific symptoms such as fever, headache, retro-orbital pain, myalgia, sore throat, nausea and vomiting were recorded.

**Table 1.** *Laboratory and clinical results following second dengue infection*

| Enrollee ID | Sex | Age | Secondary Infection | | | | | | | | | | | | | | Diagnosis |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Lab results | | | | | | | | | | Clinical manifestations | | | | |
| | | | PRNT 50% Titer | | | | IgM ELISA | | HI | | RT-PCR | Virus isolation | Symptoms and Sign | Haemorrhagis Tendencies | Thrombo-cytopeniae | Plasma leakage | Circulatory failure | |
| | | | Sero-types | Pre-illness | Acute | Conv. | Acute | Conv. | Acute | Conv. | | | | | | | | |
| 730 | M | 42 | DENV-1<br>DENV-2<br>DENV-3<br>DENV-4 | 187<br><10<br>12<br><10 | <10<br>39<br>13<br><10 | >25000<br>1463<br>6870<br>56 | 0.5 | 4.8 | <10 | 640 | Negative | Negative | Fever, Headache, Myalgia, Coryza, Nausea, Vomiting, Diarrhea, Leukopenia, | No | Yes | No | No | Dengue fever |
| 1793 | F | 29 | DENV-1<br>DENV-2<br>DENV-3<br>DENV-4 | <10<br>359<br>12<br>11 | <10<br>165<br>10<br><10 | 3402<br>>1000*<br>6024<br>490 | 0.7 | 10.9 | 160 | 10240 | DENV-3 | Negative | Headache, Retro- orbital, Pain, Myalgia, Sore-throat, Nausea, Vomiting, | Yes | No | No | No | Dengue fever |
| 2159 | M | 38 | DENV-1<br>DENV-2<br>DENV-3<br>DENV-4 | 10<br>215<br><10<br><10 | <10<br>145<br><10<br><10 | 139<br>3337<br>301<br>493 | 0.17 | 1 | <10 | 2560 | DENV-4 | DENV-4 | Fever, Headache, Myalgia, Nausea | No | No | No | No | Dengue fever |
| 2119 | F | 30 | DENV-1<br>DENV-2<br>DENV-3<br>DENV-4 | <10<br>124<br>99<br>29 | <10<br>133<br><10<br><10 | 567<br>7404<br>1029<br>5414 | 0.2 | 1.1 | 10 | 2560 | Negative | DENV-4 | Fever, Headache, Retro- Orbital, Pain, myalgia, nausea, vomiting, abdominal pain | Petechiae, Nose bleeding, Gum bleeding, Tourniquet test | Yes | Pleural Effusion, Ascites, hypo proteinemia | No | Dengue haemorrhagic fever gr.2 |

*No plaques were detected even in the highest dilution (1:1000)

**Table 2.** Laboratory and clinical results following third dengue infection

| Enrollee ID | Sex | Age | Secondary Infection | | | | | | | | | | | | | | Diagnosis |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Lab results | | | | | | | | | Clinical manifestations | | | | | |
| | | | PRNT 50% Titer | | | | IgM ELISA | | HI | | RT-PCR | Virus isolation | Symptoms and Sign | Haemorrhagis Tendencies | Thrombo-cytopeniae | Plasma leakage | Circulatory failure | |
| | | | serotypes | Pre-illness | Acute | Conv. | Acute | Conv. | Acute | Conv. | | | | | | | | |
| 730 | M | 42 | DENV-1<br>DENV-2<br>DENV-3<br>DENV-4 | 1316<br>140<br>138<br>13 | 809<br>76<br>137<br>18 | 8124<br>5537<br>>1000*<br>127 | 0.48 | 1.99 | 40 | 1280 | Negative | Negative | Fever, headache, retro-orbital pain, myalgia, nausea, leukopenia | No | No | No | No | Dengue fever |
| 1793 | F | 29 | DENV-1<br>DENV-2<br>DENV-3<br>DENV-4 | 20<br>681<br>202<br>80 | 77<br>764<br>172<br>137 | >45000<br>16750<br>>1000*<br>2126 | 1.3 | 9.3 | 160 | 2560 | Negative | Negative | Fever, headache, retro-orbital pain, myalgia, sore-throat, nausea, vomitting | Tourniquet test | Yes | No | No | Dengue fever |
| 2159 | M | 38 | DENV-1<br>DENV-2<br>DENV-3<br>DENV-4 | 198<br>271<br>99<br>64 | 51<br>505<br>57<br>61 | >60000<br>>1000*<br>>60000<br>>40000 | 0.19 | 1.1 | 80 | 5120 | DENV-3 | Negative | Fever, headache, retro-orbita pain, myalgia, sore-throat, nausea | No | No | No | No | Dengue fever |
| 2119 | F | 30 | DENV-1<br>DENV-2<br>DENV-3<br>DENV-4 | 75<br>528<br>36<br>35 | <10<br>345<br><10<br><10 | 10720<br>>1000*<br>12103<br>4146 | 0.4 | 0.8 | 80 | 5120 | DENV-3 | DENV-3 | Fever, headache, myalgia, nausea, vomitting | Tourniquet test | Yes | No | No | Dengue fever |

*No plaques were detected even in the highest dilution (1:1000)

The study volunteer also exhibited a positive tourniquet test, haemorrhagic tendencies and thrombocytopenia. Since there was neither plasma leakage nor circulatory failure the final diagnosis was DF (Table 1). The third infection with DENV-1 was milder and consequently no hospitalization was required as the patient was managed in the outpatient clinic.

A third subject, a 38-year-old male had neutralizing antibodies to DENV-2 ($PRNT_{50}$ titer:215) a month prior to his second infection with DENV-4 (confirmed by RT-PCR, virus isolation and neutralizing antibody profile). Eighteen months later, this individual experienced a DENV-3 infection as identified by RT-PCR. Both the DENV-4 and DENV-3 infections resulted in a clinically mild disease (DF). The symptoms reported were fever, headache, retro-orbital pain, myalgia, nausea and sore throat (Tables 1 and 2).



The fourth study volunteer, a 30-year-old female, demonstrated neutralizing antibodies against DENV-2 and DENV-3 and a lower titer against DENV-4 two months prior to a DENV-4 infection that was documented by the recovery of the virus from the acute serum sample. Eleven months later, the volunteer was infected by a DENV-3 virus as evidenced by RT-PCR and virus isolation. The neutralizing antibody profile in these two episodes was in accordance with the infecting serotypes. Clinical symptoms, haemorrhagic tendencies and thrombocytopenia were reported in both episodes; however, plasma leakage, as noted by pleural effusion, ascites and hypoproteinemia, was only detected after the second infection. In this patient, the second infection was diagnosed as DHF grade II and the third as DF (Tables 1 and 2).

Previously, sequential dengue infections were recorded in 1971 in an individual from India [5]. In this instance, the first infection was in 1960 by a DENV-4 virus, followed by DENV-1 one year later and DENV-2 in 1969. All the infections manifested as a mild disease without haemorrhagic tendencies. However, dengue immune status prior to each episode of illness was unknown. Furthermore, the interval between the second and third infections was very long (8 years), which may suggest that cross-immunity to DENV-2 had disappeared.

Heterologous secondary dengue infection is often associated with more severe manifestations (DHF including dengue shock syndrome-DSS). In our case series, one of the four secondary infections resulted in DHF grade II. The enrollee was infected by DENV-4 despite having low-level neutralizing antibody to this serotype ($PRNT_{50}$ titer 29). In the other three cases, pre-illness neutralizing antibodies to the infecting serotypes were not detected. All tertiary dengue infections resulted in milder diseases. Pre-illness neutralizing antibodies to the infecting serotypes ranged from 36 to 99.

Pre-illness, acute and convalescent PRNT data from episodes with confirmed infecting serotypes (by RT-PCR or virus isolation) also showed that the highest neutralizing titers among convalescent sera may indicate that the previous infecting serotypes and the current infecting serotypes resulted in the second highest neutralizing titers in the convalescent sera. Our work supports the original antigenic sin theory first proposed by Halstead [6].

These data support early studies that imply that the presence of neutralizing antibodies against additional dengue viruses clearly does not protect against subsequent dengue infections.

## ACKNOWLEDGMENT

The authors thank Chairin Ma'roef, Erlin Listiyaningsih, Drh. Susana Widjaja and Dr Ardini Raksanagara for their assistance in virus isolation, RT-PCR, serology assays and clinical data collection. We are grateful to Dr Timothy H. Burgess for critical review. The opinions offered within represent those of the authors and do not reflect the official policy of the US Department of Defense or the Department of the Navy.

## References

1. George R (1987) Dengue haemorrhagic fever in Malaysia: a review. Southeast Asian J Trop Med Public Health 18: 278-283.
2. Suroso T (1997) Perkembangan Kebijaksanaan Upaya Pemberantasan Penyakit Demam Berdarah Dengue (DBD) di Indonesia (1968-1996). Workshop on Emerging Infectious Disease in Sawangan, East Java.
3. Porter KR, Beckett CG, Kosasih H, Tan RI, Alisjahbana B, et al. (2005) Epidemiology of dengue and dengue hemorrhagic fever in a cohort of adults living in Bandung, West Java, Indonesia. Am J Trop Med Hyg 72: 60-66.
4. World Health Organization (1999) Prevention and control of dengue and dengue haemorhagic fever. comprehensive guidelines New Delhi: Regional Office for South-East Asia SEARO Regional Publication No. 29.
5. Myers RM, Varkey MJ (1971) A note on sequential dengue infection, presumptive and proved, with report of an instance of a third proved attack in one individual. Indian J Med Res 59: 1231-1236.
6. Halstead SB, Rojanasuphot S, Sangkawibha N (1983) Original antigenic sin in dengue. Am J Trop Med Hyg 32: 154-156.

# Chapter 5

## Comparison of the hemagglutination inhibition test and IgG ELISA in categorizing primary and secondary dengue infections based on the plaque reduction neutralization test

*submitted to PLoS One*

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## ABSTRACT

Secondary dengue infection by a heterotypic serotypes is associated with severe manifestations of disease, i.e. dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The World Health Organization (WHO) has recommended criteria based on the hemagglutination inhibition (HI) assay to distinguish between primary and secondary dengue infections. Since the HI assay has practical limitations and disadvantages, we compared the WHO HI criteria with criteria based on an IgG enzyme-linked immunosorbent assay (ELISA) assay, using a plaque reduction neutralization test (PRNT) as the gold standard. Both HI and IgG ELISA criteria performed strongly (16/16) in determining primary infection. However, to determine secondary infections, the IgG ELISA criteria performed better (72/73) compared to the WHO HI criteria (23/73).


## INTRODUCTION

Dengue virus is a global concern, with increasing incidence especially in endemic areas like Southeast Asia, South America and the Pacific. Recent analysis based on the geographical distribution of the disease, estimates 250-500 million infections annually, which is three times higher than the estimation of the World Health Organization [1-3].

Dengue virus (DENV) belongs to the family *Flaviviridae*, genus *Flavivirus*, which consists of four antigenically distinct serotypes (DENV-1, -2, -3 and -4). Infection with any one of the four serotypes can be asymptomatic or result in a wide range of clinical manifestations from dengue fever (DF), mild to severe dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS) which is often fatal. Besides viral virulence and host background (immune, genetic and nutritional), severe dengue is often associated with secondary infection [4]. Secondary infections, which are common in endemic areas such as Indonesia [5-8], comprised approximately 80-90% of the DHF/DSS cases. It is therefore important to distinguish between primary and secondary infection. The 1997 World Health Organization (WHO) guidelines on dengue suggested criteria based on hemagglutination inhibition (HI) titers to distinguish between primary

and secondary infection. While the accuracy of the HI criteria in distinguishing between primary and secondary infections has been questioned [9-14], this criteria is still used in studies that would like to analyze the relationship between primary or secondary infections with the severity of disease as well as studies conducted to develop other serological methods [9,10,15-17]. Several methods based on enzyme-linked immunosorbent assay (ELISA), which is easier and faster to perform, have been proposed as alternative methods to distinguish primary and secondary infections, for example, by measuring IgG antibodies; the avidity titer of IgG; calculating the ratio of IgM/IgG; or the absence of IgG in acute specimens [11,12,14,18,19]. However, none of these studies utilized another serological assay as the gold standard. In this study, we compared the utility of the HI test and IgG ELISA in discriminating primary and secondary infections using acute and convalescent specimens that had been tested by a plaque reduction neutralization test (PRNT). In addition, we also propose modified HI criteria to distinguish between primary and secondary infections.



## MATERIALS AND METHOD

### Sample collection

A total of 178 acute and convalescent sera from 89 confirmed dengue cases were used in this study. These sera were collected as a part of two dengue studies in Bandung. These studies were approved by the Institutional Review Board of United Stated Navy Medical Research Unit (US-NAMRU)#2 (N2.2006.0001 and N2.2004.0010, respectively) in compliance with all U.S. Federal Regulations governing the protection of human subjects and at the National Institute of Health Research and Development, Ministry of Health, Indonesia (KS.02.01.2.1.2776 and KS.02.01.2.1.3461, respectively). Acute sera were taken when study participants came to health centers or hospitals, while convalescent sera were obtained at least seven days later or when the participants were discharged from the hospitals.

## Dengue diagnosis

A dengue infection was confirmed when : 1). DENV was isolated from tissue culture or DENV RNA was detected by the reverse-transcriptase polymerase chain reaction or 2). when the acute sample was positive for IgM and paired sera demonstrated increasing IgG titers and/or a four-fold rise in HI titers.

The HI test was performed as previously described [20]. A preliminary experiment comparing the performance of all four dengue antigens in the HI test did not show any difference in their ability to determine recent infection or in differentiating primary and secondary infections (data not shown). Hence, only dengue-3 antigen was used for HI tests throughout the rest of this study. Specimens with detectable HI antibodies at 1:10 or higher dilutions were confirmed as positive. If no antibody was detected at the lowest dilution (1:10), the specimen was confirmed as negative. Any case with a convalescent HI antibody titer higher than 1280 was classified as a secondary infection [21].

IgG ELISA was performed using a commercial dengue IgG ELISA kit (Dengue IgG ELISA, Focus Technologies, Cypress, CA, USA) according to the manufacturer's instructions. The index value was calculated by dividing the sample optical density (OD) with the mean of calibrator OD. Specimens with an IgG index value <1 were confirmed as negative and those with an IgG index value >1 were confirmed as positive. When IgG was negative in the acute specimen the case was classified as a primary infection.

PRNT was performed using the following reference strains: DENV-1 (16001), DENV-2 (16682), DENV-3 (16562), and DENV-4 (1036). In brief, 500 plaque forming units per milliliter of dengue virus were incubated with serial 4-fold diluted serum from 1:10 to 1:10,240. A suspension of the baby hamster kidney (BHK) cells was used to detect the virus reduction following previously described methods [22]. The $PRNT_{50}$ titer was calculated using a log probit regression analysis by SPSS (SPSS Inc., Chicago, IL). A sample was confirmed as negative for neutralizing antibodies when the $PRNT_{50}$ values were lower than 10 against all dengue serotypes. A sample was confirmed as positive for neutralizing antibodies when

$PRNT_{50}$ titers were 10 or greater for at least one dengue serotype. Any case with seroconversion of neutralizing antibodies was classified as a primary infection. Any case with detectable neutralizing antibodies in the acute specimen was confirmed as a secondary infection.

### Statistical analysis

Statistical analysis was performed using STATA version 9 (STATA Corporation, TX). Comparison between two assays was analyzed using McNemar's test.

## RESULTS

Subject demographics and specimen characteristics categorized by primary and secondary infection according to PRNT results are presented in Table 1. Subjects with primary infections were slightly younger that those subjects with secondary infections, but there was no difference in the gender distribution, acute sample collection day, convalescent sample collection day, or the interval between acute and convalescent sample collection.

**Table1.** Subject and Sample characteristics

| | Subjects | | Samples | | |
|---|---|---|---|---|---|
| | **Age (years)** | **Sex (Male, %)** | **Acute (days of onset)** | **Convalescent (days of onset)** | **A-C interval (days)** |
| Primary | 26±9 (7-42) | 13 (65) | 2.5±1.7 (1-7) | 18.5±5.9 (9-32) | 15.9±5.6 (7-30) |
| Secondary | 36±8 (9-53) | 47(65) | 2.1±1.3 (1-7) | 15.3±4.4 (6-28) | 13.2±3.9 (4-26) |
| Total | 34±9 (7-53) | 60 (65) | 2.2±1.4 (1-7) | 15.9±4.8 (6-32) | 13.7±4.4 (4-30) |

* Mean +/- SD (range)

## HI and IgG ELISA performances compared to PRNT

First, we sought to examine the sensitivity and specificity of the IgG ELISA and HI assays in comparison to the PRNT. Based on PRNT results, 162 of 178 specimens were positive for dengue neutralizing antibodies and 16 specimens (all acute) were negative. Among the 162 positive samples, IgG ELISA results were positive for 161 (99.4%) samples and HI results were positive for 153 (94.4%) samples. Among the 16 specimens negative by PRNT, IgG ELISA results were also negative for these samples whereas HI antibodies results were negative in only 10 (62.5%) samples. Thus, while the sensitivities between ELISA and HI were similar compared to PRNT, the IgG ELISA was significantly more specific than the HI assay ($p<0.01$).

## Distinguishing between primary and secondary infections

We next aimed to determine the performance of the IgG ELISA and HI assays in distinguishing between primary and secondary infections using PRNT results as the gold standard. According to the PRNT results, of the 89 dengue cases included in this study, 16 cases were categorized as primary infections and 73 cases were categorized as secondary infections. Of 16 cases classified as primary infections, classification by HI and IgG ELISA concurred in all of these cases. IgG ELISA also identified 72 of 73 (98.6%) secondary cases, whereas HI was only able to detect 23 of the 73 (31.5%) secondary cases. The difference between the two was significant ($p<0.01$). When convalescent HI titers were compared to PRNT classification 11 cases with HI titers ≤80 were primary infections, 46 cases with HI titers ≥1280 were secondary, 32 cases with HI convalescent titers between 160 and 640, PRNT results only confirmed primary infections in five (15.6%) cases (Table 2).

**Table 2.** Comparison of HI test and PRNT on Convalescent-phase samples

| HI Titer | Cases | PRNT | | | |
|---|---|---|---|---|---|
| | | Primary infections | | Secondary infections | |
| | | Cases | Percentage | Cases | Percentage |
| ≤80 | 11 | 11 | 100 | 0 | 0 |
| 160 | 4 | 1 | 25 | 3 | 75 |
| 320 | 14 | 3 | 21.4 | 11 | 78.6 |
| 640 | 14 | 1 | 7.1 | 13 | 92.9 |
| 1280 | 23 | 0 | 0 | 23 | 100 |
| ≥2560 | 23 | 0 | 0 | 23 | 100 |



To determine if we could better categorize the cases with convalescent HI titers between 160 and 640, we considered HI results from the acute samples of these cases. For this analysis, cases with convalescent HI titers between 160 and 640 and negative acute sample HI results were considered primary cases and cases with convalescent HI titers between 160 and 640 and positive acute sample HI results were considered secondary cases. Using this classification algorithm, 24 of 32 (75%) cases with convalescent HI titers between 160 and 640 were classified in concordance with PRNT results. We also considered using only acute sample HI results for classification by defining cases with no acute HI antibodies as primary infections. Using this criteria, 64/73 secondary cases and 10/16 primary cases were classified in concordance with PRNT results.

Table 3 summarizes the percentage of cases classified in concordance with PRNT results as primary or secondary infections using all of the criteria examined in this study. Cases classified according to an acute sample IgG ELISA results had the highest concordance with PRNT based classification while criteria that took into account convalescent and acute HI results had the next best concordance with PRNT classification.

**Table 3.** Percent concordance with PRNT case classification using ELISA IgG, HI (WHO 1997) or modified HI criteria

| | IgG ELISA | HI (WHO 1997) | Modified HI-1[a] | Modified HI-2[b] |
|---|---|---|---|---|
| 1st infection | 16/16 (100)[1] | 16/16(100)[2] | 10/16(62.5)[1,2] | 13/16(81.3) |
| 2nd infection | 72/73(98.6)[3,4] | 23/73(31.5)[3,5,6] | 64/73(87.7)[4,5] | 68/73(93.2)[6] |

Criteria to determine primary infection//secondary infections:

[a] 1st infection if HI Abs negative in acute specimens

[b] 1st infection if convalescent HI Abs ≤ 80, or if convalescent HI Abs are 160, 320, or 640 and HI Abs negative in acute specimen

[1,2,3,4,5,6] statistically significant

## DISCUSSION

Serology assays are the most applicable and affordable confirmatory test for dengue infections in third world countries where dengue is commonly endemic. In addition, WHO has recommended IgM Antibody Capture (MAC) ELISA, HI and PRNT to determine primary and secondary infections [21]. Compared with MAC ELISA and PRNT, HI has long been the most widely used serology assay. However, similar to previous reports, we showed that HI underestimated secondary infections when the WHO recommended cut-off was applied [13,14]. By applying modified cut-off criteria and both convalescent and acute sample HI results, the accuracy in determining secondary infection increased from 31.5% to 93.2%, while the accuracy in determining primary infections decreased from 100% to 81.3% (13/16).

Nevertheless, compared to the HI test, the commercially available ELISA kit is simpler and faster than the HI test. It is also easier to obtain than HI antigens in some countries such as Indonesia. Although several kinds of ELISA evaluation

(avidity, ratio IgM/IgG) have been compared with HI and have been determined to be more reliable than HI tests, these studies did not use a third assay as a reference [12,14,18,19]. By using PRNT as the gold standard, our study has confirmed results from previous studies that ELISA is superior to HI in determining primary and secondary infections.

Our study was similar to de Souza et al. 2007 in regards to the IgG ELISA kit used (Focus Diagnostics) and the simple criteria for determining primary infection or secondary infection - the absence or presence of IgG antibodies in the acute specimens. As other IgG ELISA kits may have different sensitivities and specificities, this criteria should only be used after the sensitivity and specificity of these kits are assessed. Compared to other methods that used IgG ELISA and showed similar performance, such as IgM/IgG ratio and avidity index, our method is simpler and more cost-effective.



In conclusion, considering the limitation of HI criteria in discriminating primary and secondary infections, we recommend the use of the absence or presence of IgG antibodies in acute specimens as measured by IgG ELISA as the criteria. HI may still be used, however, the criteria for interpretation should be revised. We propose that HI convalescent titers ≤80 should be classified as primary infections, HI convalescent titers ≥1280 should be classified as secondary infections and for cases with convalescent HI titers between 160 and 640, the acute samples should be evaluated. If the acute sample does not have any detectable HI antibodies, the case should be classified as a primary infection. If the acute sample does have detectable HI antibodies, the case should be classified as a secondary infection. The performance of acute IgG ELISA criteria and the proposed HI criteria are equally good.

## ACKNOWLEDGEMENT

We would like to thank all the volunteers, dengue research teams at the factories and Hasan Sadikin Hospital, National Institute Health Research and Development, Ministry of Health and NAMRU#2 for their dedication in dengue research, and

to Kendra Chittenden from the USAID and INA-RESPOND for their support and assistance.

## References


1. WHO (2012) Dengue and Severe Dengue. World Health Organization.
2. Egger JR, Coleman PG (2007) Age and clinical dengue illness. Emerg Infect Dis 13: 924-925.
3. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, et al. (2013) The global distribution and burden of dengue. Nature 496: 504-507.
4. Guy B, Almond JW (2008) Towards a dengue vaccine: progress to date and remaining challenges. Comp Immunol Microbiol Infect Dis 31: 239-252.
5. Suwandono A, Kosasih H, Kusriastuti R, Harun S, Ma'roef C, et al. (2006) Four dengue virus serotypes found circulating during an outbreak of dengue fever and dengue haemorrhagic fever in Jakarta, Indonesia, during 2004. Trans R Soc Trop Med Hyg 100: 855-862.
6. Sam SS, Omar SFS, Teoh B-T, Abd-Jamil J, AbuBakar S (2013) Review of Dengue Hemorrhagic Fever Fatal Cases Seen Among Adults: A Retrospective Study. PLoS Negl Trop Dis 7: e2194.
7. Ngwe TM, Thant KZ, Inoue S, Kurosawa Y, Lwin YY, et al. (2013) Serological characterization of dengue virus infections observed among dengue hemorrhagic fever/dengue shock syndrome cases in Upper Myanmar. J Med Virol 85: 1258-1266.
8. Sharp TM, Hunsperger E, Santiago GA, Munoz-Jordan JL, Santiago LM, et al. (2013) Virus-specific differences in rates of disease during the 2010 Dengue epidemic in Puerto Rico. PLoS Negl Trop Dis 7: e2159.
9. Miagostovich MP, Nogueira RM, dos Santos FB, Schatzmayr HG, Araujo ES, et al. (1999) Evaluation of an IgG enzyme-linked immunosorbent assay for dengue diagnosis. J Clin Virol 14: 183-189.
10. Shu PY, Chen LK, Chang SF, Yueh YY, Chow L, et al. (2003) Comparison of capture immunoglobulin M (IgM) and IgG enzyme-linked immunosorbent assay (ELISA) and nonstructural protein NS1 serotype-specific IgG ELISA for differentiation of primary and secondary dengue virus infections. Clin Diagn Lab Immunol 10: 622-630.
11. Matheus S, Deparis X, Labeau B, Lelarge. J, Morvan J (2005) Use of four dengue virus antigens for determination of dengue immune status by enzyme-linked immunosorbent assay of immunoglobulin G avidity. J Clin Microbiol 43: 5784-5786.
12. Matheus S, Deparis X, Labeau B, Lelarge J, Morvan J, et al. (2005) Discrimination between primary and secondary dengue virus infection by an immunoglobulin G avidity test using a single acute-phase serum sample. Journal of clinical microbiology 43: 2793-2797.
13. Graham RR, Juffrie M, Tan R, Hayes CG, Laksono I, et al. (1999) A prospective

seroepidemiologic study on dengue in children four to nine years of age in Yogyakarta, Indonesia I. studies in 1995-1996. Am J Trop Med Hyg 61: 412-419.

14. A-nuegoonpipat A, Prakong S, Sa-ngasang A, Chanama S, Sawanpanyalert P, et al. (2006) Comparison between Haemagglutination Inhibition (HI) Test and IgM and IgG-capture ELISA in Determination of Primary and Secondary Dengue Virus Infections. Dengue Bulletin 30: 141-145.
15. Lam SK, Devine PL (1998) Evaluation of capture ELISA and rapid immunochromatographic test for the determination of IgM and IgG antibodies produced during dengue infection. Clin Diagn Virol 10: 75-81.
16. Becerra A, Warke RV, de Bosch N, Rothman AL, Bosch I (2008) Elevated levels of soluble ST2 protein in dengue virus infected patients. Cytokine 41: 114-120.
17. Sriprom M, Pongsumpun P, Yoksan S, Barbazan P, Gonzalez J, et al. (2003) Dengue haemorrhagic fever in Thailand, 1998-2003: primary or secondary Infection. Dengue Bulletin 27: 39-45.
18. Vaughn DW, Nisalah A, Solomon T, Kalayanarooj S, Nguyen MD (1999) Rapid serologic diagnosis of dengue virus infection using a commercial capture ELISA that distinguishes primary and secondary infections. Am J Trop Med Hyg 60: 693-698.
19. de Souza VA, Tateno AF, Oliveira RR, Domingues RB, Araujo ES, et al. (2007) Sensitivity and specificity of three ELISA-based assays for discriminating primary from secondary acute dengue virus infection. J Clin Virol 39: 230-233.
20. Clarke DH, Casal J (1958) Technique for Hemagglunation and Hemagglunation Inhibition with Arthropod Borne Virus. Am J Trop Med Hyg 7: 561-567.
21. WHO (1997) Dengue haemorrhagic fever: diagnosis, treatment, prevention, and control. Genevea, Switzerland: World Health Organization.
22. Morens DM, Halstead SB, Repik PM, Putvatana R, Raybourne N (1985) Simplified plaque reduction neutralization assay for dengue viruses by semimicro methods in BHK-21 cells: comparison of the BHK suspension test with standard plaque reduction neutralization. J Clin Microbiol 22: 250-254.

# Chapter 6

## The diagnostic and prognostic value of dengue non-structural 1 antigen detection in a hyper-endemic region in Indonesia

*PLoS One, 2013*

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## ABSTRACT

As dengue fever is undifferentiated from other febrile illnesses in the tropics and the clinical course is unpredictable, early diagnosis is important. Several commercial assays to detect dengue NS1 antigen have been developed; however, their performances vary and data is lacking from hyper-endemic areas where all four serotypes of dengue are equally represented. To assess the sensitivity of the Bio-Rad Platelia Dengue NS1 antigen assay according to virus serotype, immune status, gender, and parameters of severe disease, acute sera from 220 individuals with confirmed dengue and 55 individuals with a non-dengue febrile illness were tested using the Bio-Rad Platelia Dengue NS1 antigen assay. The overall sensitivity of the NS1 ELISA was 46.8% and the specificity was 100%. The sensitivity in primary infections was significantly higher than in secondary infections (100% vs. 35.7%). In secondary infections, the sensitivity of NS1 detection was highest in DENV-3 (47.1%), followed by DENV-1 (40.9%), DENV-2 (30%) and DENV-4 (27%) infections. NS1 was less frequently detected in sera with high titers of HI antibodies or in acute samples from patients whose pre-illness sera showed neutralizing antibodies to more than one serotype. The detection of NS1 was higher in females, severe cases, and individuals with lower platelet counts (<100,000/mm$^3$). While the overall sensitivity of this NS1 ELISA is poor, our data suggest that in secondary infections, detection may be predictive of a more severe illness.


## INTRODUCTION

Dengue fever (DF) is a major public health problem with 50 million annual cases worldwide. It has been reported in more than 100 countries and continues to spread to previously unaffected regions [1]. DF is caused by dengue viruses (DENV), which consist of four serotypes: DENV-1, DENV-2, DENV-3 and DENV-4. Infection with any serotype usually results in asymptomatic infection or mild non-specific febrile illness, but in a subset of patients, severe disease develops, characterized by a transient capillary leakage syndrome (dengue hemorrhagic fever/ DHF) [2]. This unpredictable disease course and the need to differentiate

DF from other causes of fever make an early and sensitive diagnosis of acute dengue virus infection important.

Non-structural 1 protein (NS1) is encoded by the virus and secreted in a soluble form [3]. Several commercial assays to detect dengue NS1 have been developed recently [4]. Advantages of NS1 tests are: NS1 is detected early in disease, several days prior to the appearance of anti-dengue IgM antibodies [5], and the test is inexpensive, easy, and fast. Moreover, NS1 levels early in dengue disease have been correlated with disease severity [6], suggesting that NS1 tests may also have prognostic value.

Previous studies evaluating the diagnostic value of NS1 antigen assays have had varying results [4,7] (Table S1). Several factors may account for this : proportion of primary vs. secondary infections [4,8-11], timing of sample collection [4,7,8,12,13], infecting serotype [4,7,8,13,14], viremia levels [6,13,15,16] and severity of illness [6,7,15]. A low sensitivity has been reported in secondary infections possibly due to immune-complex formation of NS1 and pre-existing antibodies [17]. This may especially be important as the proportion of secondary infections is expected to rise worldwide [18]. Although studies evaluating the NS1 assays were conducted in hyper-endemic areas of Southeast Asia or South America, in several studies, all four dengue serotypes were not represented equally [4,7,8,10,13,15,16,19,20] or evaluated [11,21]. In others, the percentage of primary infections was too high to represent the situation in hyper-endemic areas [9,11,12,15,20]. Additionally, only a few studies evaluated the association between NS1 detection and clinical severity [4,6,7,15,21]. The objectives of the study were: 1) Determine diagnostic performance of the Bio-Rad Platelia Dengue NS1 antigen assay in a region where all dengue serotypes circulate year round and clinical severity could be evaluated 2) Assess the sensitivity of this assay in secondary cases according to pre-illness and acute dengue immune status 3) Determine the sensitivity of this assay according to clinical severity.

# MATERIALS AND METHODS

## Ethics Statement

All samples were obtained after written informed consent through studies approved by the Institutional Review Board of NAMRU#2 (N2.2006.0001, N2.2004.0010, N2.2004.0008) and at the National Institute of Health Research and Development, Ministry of Health, Indonesia (KS.02.01.2.1.2776, KS.02.01.2.1.3461 and KS.02.01.2.1.2336) in compliance with all U.S. Federal Regulations governing the protection of human subjects.

## Sample characteristics

Acute sera from archived and fresh samples of 220 confirmed dengue cases and 55 non-dengue febrile illness cases were used. Samples were from previously published studies [22,23]. Study one was a prospective dengue cohort study of ~3,000 factory workers in two factories in Bandung, Indonesia from 2000-2004 and 2006-2009. Volunteers participated in serosurveys every three months and visited factory clinics when they experienced fever. Study two was a community dengue study where 15-20 people living near a confirmed dengue case were observed for two weeks when a dengue case was confirmed at the study hospital. Volunteers in study one and two were hospitalized when dengue infection was confirmed. Study three was a hantavirus surveillance study at two hospitals in Bandung, Indonesia. As clinical manifestations between dengue and hantavirus are indistinguishable, samples were screened for evidence of dengue infection. In all studies, acute sera were collected when volunteers presented at clinics or hospitals, and convalescent samples were collected during hospital discharge or 7-10 days after acute sample collection. Paired samples were tested for evidence of dengue infection as described below. Complete blood counts to detect thrombocytopenia and hematocrit measurements to look for hemoconcentration as one indicator of plasma leakage were taken daily for hospitalized patients and upon indication for outpatients. Ultrasonography to detect plasma leakage was performed on hospitalized patients in study one and two.

## Case Definitions

A dengue infection was confirmed when 1. DENV RNA was detected by RT-PCR or 2. For RT-PCR negative cases, when all of the following criteria were met: IgM positive, increasing IgG titers and a four-fold rise in hemagglutination inhibition (HI) titers. A primary or secondary dengue infection was determined by the absence or presence of IgG antibodies in the acute sample [24]. Non-dengue infection was confirmed when dengue RT-PCR was negative, and no IgM antibodies, increase in IgG titers or four-fold increase of HI antibody titers was detected. Infecting serotypes were determined by RT-PCR and neutralizing antibodies to each serotype in pre-illness sera were measured by plaque reduction neutralization test (PRNT). Dengue cases were classified as DF, DHF, and dengue shock syndrome (DSS) according to 1999 WHO guidelines [25]. Cases of DF with hemorrhagic manifestations formed a separate group (DF+HM).



## Laboratory tests

Acute serum samples were assayed for DEN virus RNA by RT-PCR [26]. Acute and convalescent sera were tested for dengue IgM, IgG and HI antibodies. Dengue IgM and IgG were detected by capture and indirect ELISA respectively according to the manufacturer's instructions (FOCUS Technologies, CA, USA). HI tests were performed as previously described [27]. Acute samples were tested with the NS1 Platelia antigen capture ELISA according to the manufacturer's instructions (BioRad, CA, USA). PRNT was conducted on pre-illness sera collected during the serosurvey in study one from 2000-2004. Three 10-fold serum dilutions were made starting at 1:10 and diluted samples were assayed as described previously [28]. The dilution that produced an 80% reduction in plaque count compared with the negative control sample was determined by probit analysis using SPSS (SPSS, Chicago, IL). The virus strains used in the assays were isolated from DEN patients in Thailand (16001 DENV-1, 16682 DENV-2, and 16562 DENV-3) and Indonesia (1036 DENV-4).

### Statistical analysis

Data were entered into Microsoft Access. Statistical analysis was performed using STATA version 9 (STATA Corporation, TX). Categorical variables between two groups were compared with a chi-square test. A p value of less than 0.05 was considered significant.

## RESULTS

### Overall and serotype specific sensitivity of the NS1 test

Samples from 275 volunteers were used, 220 confirmed dengue cases and 55 non-dengue cases. Dengue infection was confirmed by both virological and serological evidence in 195 (88.6%) cases and by serological evidence alone in 25 (11.4%) cases. Table 1 summarizes the characteristics, WHO clinical category, virological and serological data for these cases. The overall sensitivity of the assay was 46.8% (95% CI: 40.2-53.3) and the specificity was 100% (55/55) (Table 2). The sensitivity was 50.3% (95% CI: 43.3-57.3) in samples positive by RT-PCR, and 20% (95% CI: 4.3-53.7) in samples negative by RT-PCR. The sensitivity was 100% (34/34) in primary infections and 35.7% (95% CI: 28.7-42.7) in secondary infections (p=0.00). In secondary infections, no significant (p=0.38) difference was found between fresh and archived specimens (38.9%, 95%CI: 28.8-49 vs.32.6%, 95%CI: 23-42.2); however, the sensitivity by serotype varied. The highest sensitivity was observed for DENV-3 (47.1%, 95% CI: 35.2-58.9), followed by DENV-1 (40.9%, 95% CI: 20.4-61.4), DENV-2 (30%, 95% CI: 13.6-46.4) and DENV-4 (27%, 95% CI: 12.7-41.3). This difference in sensitivity was significant when comparing DENV-3 to DENV-4 (p=0.045.).

**Table 1.** Specimen characteristics

| Source of Specimens | Study 1 | Study 2 | Study 3 | Total |
|---|---|---|---|---|
| Non-dengue | 51 | 4 | 0 | 55 |
| Acute dengue | 125 | 27 | 68 | 220 |
| Mean age in years (range) | 36.5 (21-53) | 19.5 (4-47) | 24.4 (12-50) | 30.7 (4-53) |
| Sex Ratio (male:female) | 3.3:1 | 1:1.1 | 1.4:1 | 2.1:1 |
| Outpatients | 72 | 8 | 0 | 80 |
| Inpatients | 53 | 19 | 68 | 140 |
| Mean day after illness onset (range) | 2.7 (1-7) | 2 (1-7) | 5.1 (2-7) | 3.5 (1-7) |
| Clinical diagnosis (%) | | | | |
| *dengue fever (DF)* | 84 | 9 | 33 | 126 (57.3) |
| *DF with hemorrhagic manifestations* | 8 | 2 | 22 | 32 (14.5) |
| *dengue hemorrhagic fever grade I* | 18 | 6 | 5 | 29 (13.2) |
| *dengue hemorrhagic fever grade II* | 15 | 8 | 8 | 31 (14.1) |
| *dengue shock syndrome* | 0 | 2 | 0 | 2 (1) |
| Infecting serotype (%) | | | | |
| DENV-1 | 23 | 6 | 6 | 35 (15.9) |
| DENV-2 | 19 | 5 | 18 | 42 (19.1) |
| DENV-3 | 35 | 6 | 40 | 81 (36.8) |
| DENV-4 | 32 | 1 | 4 | 37 (16.8) |
| Unknown | 16 | 9 | 0 | 25 (11.4) |
| Type of infection (%) | | | | |
| Primary | 16 | 5 | 13 | 34 (15.4) |
| Secondary | 109 | 22 | 51 | 182 (82.7) |
| Unknown | 0 | 0 | 4 | 4 (1.8) |

**Table 2.** Sensitivity of the NS1 antigen test according to infection status

| Type of infection | Infecting serotypes | | | | | TOTAL |
|---|---|---|---|---|---|---|
| | DENV-1 | DENV-2 | DENV-3 | DENV-4 | Unknown* | |
| Primary | 13/13(100) | 11/11(100) | 10/10(100) | 0/0(0) | 0/0(0) | 34/34(100)[1] |
| Secondary | 9/22 (40.9) | 9/30 (30) | 32/68(47.1)[2] | 10/37(27)[2] | 5/25(20) | 65/182(35.7)[1] |
| Unknown# | 0/0(0) | 1/1(100) | 3/3(100) | 0/0(0) | 0/0(0) | 4/4(100) |
| Total | 22/35(62.9) | 21/42(50) | 45/81(55.6) | 10/37(27) | 5/25(20) | 103/220(46.8) |

*PCR negative, #IgG results not available,

[1]statistically significant difference (p=0,00)

[2]statistically significant difference (p=0.04)

## NS1 sensitivity according to dengue immune status

To determine if the sensitivity of the NS1 test varied by dengue immune status, dengue HI titers were determined on all acute samples and PRNTs were performed on pre-illness (one to two months prior to dengue infection) sera from the 47 patients enrolled in the first phase of study one. The sensitivity was significantly ($p<0.05$) lower in acute samples with high HI titers (≥1280) compared to samples with lower or undetectable titers (Table 3A). Among the 47 samples tested by PRNT, neutralizing antibodies to more than one serotype were detected in 32 cases and NS1 was only positive in one acute specimen, giving a sensitivity of 3.1%. In contrast, in patients with pre-illness neutralizing antibodies to only one serotype, the sensitivity of the NS1 test was 60% (9/15) (Table 3B). Among these nine NS1 positive cases, eight had pre-illness neutralizing antibodies to DENV-2 and one to DENV-1, whereas among the six negative cases, four had neutralizing antibodies to DENV-1, one to DENV-3 and one to DENV-2. The infecting serotypes in eight of the nine positive cases were DENV-1 (2), DENV-3 (2) and DENV-4 (4).

**Table 3.** Sensitivity of the NS1 antigen test according to dengue immune status

| A. Acute specimen HI titer (n=179) | |
|---|---|
| HI titer | Sensitivity |
| < 10 | 26/32(81.3%)[1] |
| 10 – 80 | 29/72 (40.3%) |
| 160 – 640 | 18/40 (45%) |
| ≥1280 | 7/35 (22%)[1] |
| B. Presence of pre-illness neutralizing antibodies* (n=47) | |
| Number of serotypes | Sensitivity |
| 1 | 9/15[4] (60%) |
| more than 1 | 1/32[4] (3.1) |

[1]significantly different from the other groups
*plaque reduction neutralizing antibodies (80%)
[4]significantly different from each other

## NS1 sensitivity according to disease severity and gender

As the sensitivity of the NS1 assay was 100% in primary infections, only secondary infections were included in this analysis. The sensitivity among DF cases (25%, 95% CI: 16.7-33.3) was significantly lower compared to DF+HM cases (55%, 95% CI: 33.2-76.8) and DHF I and DHF II cases (50%, CI: 36.9-63.1). Additionally, the sensitivity was higher in patients with platelet counts below 100,000/mm$^3$ at presentation (48% vs. 27.1%, p=0.00) or at time of platelet nadir (50% vs.16.2%, p=0.00). A higher sensitivity was also found in cases with spontaneous hemorrhagic manifestations, hemoconcentration or effusion, but these differences were not significant (Figure 1).

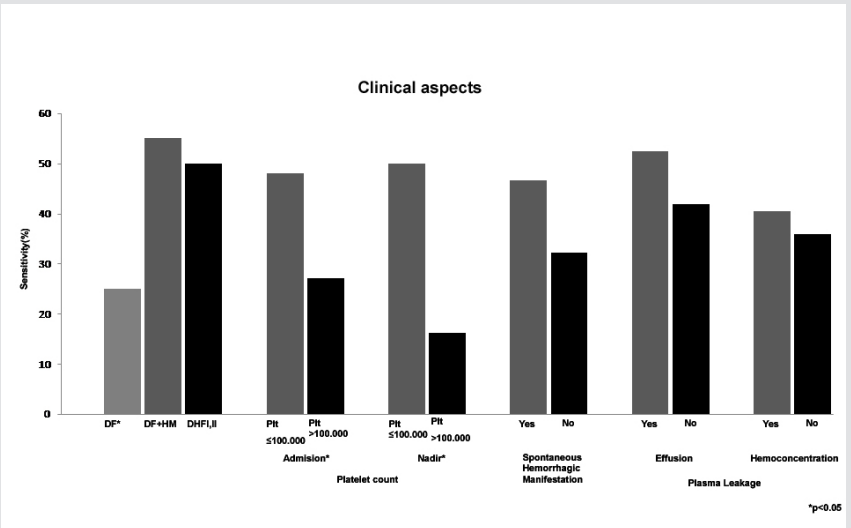


**Figure 1.** Sensitivity of the NS1 antigen test according to diagnosis category and clinical variables in secondary dengue cases.

Data from a subset of patients enrolled in study one and two in which patients were identified early, revealed that the mean and range interval between the detection of NS1 and thrombocytopenia among 28 patients was 1.8 (0-5) days, whereas the time between the detection of NS1 and evidence of plasma leakage

among 17 DHF patients was 3.3 (1-5) days. The sensitivity of the NS1 assay was significantly higher in females than males with values of 49.1% (95% CI: 36.1-62.1) and 29.6% (95% CI: 21.6-37.6) respectively (p=0.01). This gender difference remained present when analysis was restricted to those with manifestations of more severe disease (Figure 2).

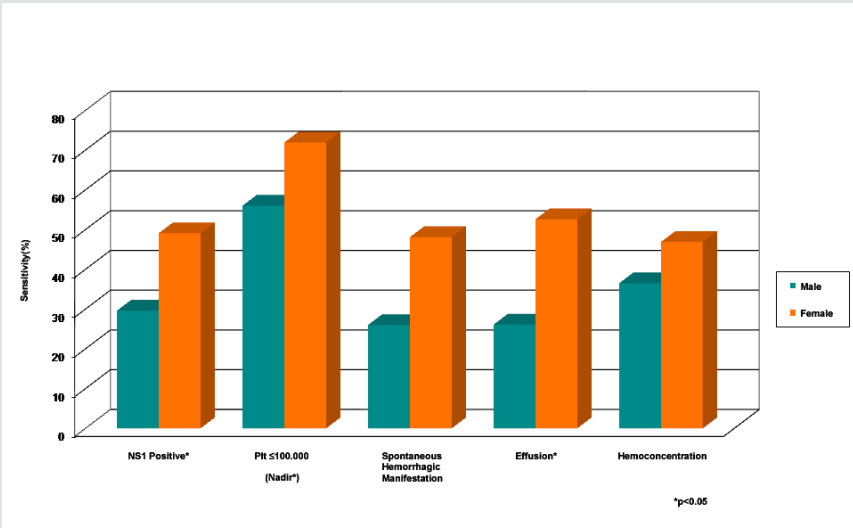


**Figure 2.** The sensitivity of the NS1 antigen test according to gender and clinical variables in secondary infections.

## DISCUSSION

NS1 tests are increasingly being used as a rapid and inexpensive diagnostic tool for acute DF. We found that its sensitivity in a dengue hyper-endemic area (Indonesia) where most infections are secondary was low with an overall sensitivity of 46.8%. In samples from primary infections, the sensitivity was 100% indicating that it is a good diagnostic tool for patients from non-endemic areas, such as in travelers [29]. Our findings are also in line with reports from other

countries that found a low sensitivity in secondary infections [4,13,20]. Apart from primary or secondary infection status, our data also suggest that sensitivity was affected by dengue immune status, infecting dengue serotype, gender and severity of illness.

Our data demonstrated poor performance of the NS1 test in acute sera with high titers of HI dengue antibodies. Further analysis showed that NS1 antigen was undetectable in nearly all acute sera from patients with pre-illness neutralizing antibodies to more than one serotype, but was detectable in more than half of those with only one serotype. These findings are in line with the hypothesis that immune-complexes are formed in secondary infections and high levels of antibodies capture and bind soluble NS1 (sNS1) resulting in a reduction of circulating NS1 [17]. Therefore, the use of a NS1 assay in areas where post-secondary dengue infection is common [30,31] has limited value. The serotype of a previous dengue infection may also influence the sensitivity of NS1 detection in secondary infections as we found the sensitivity was significantly higher in acute samples from patients with pre-illness neutralizing antibodies to DENV-2 (88.9%) compared to DENV-1 (11.1%). It has been reported that in comparison to DENV-1 and DENV-3, DENV-2 infections result in much lower concentrations of sNS1 [32]. Thus, during primary DENV-2 infections, the amount of sNS1 produced may be unable to generate sufficient NS1 antibodies to form immune-complexes with sNS1 in secondary infections. The influence of pre-existing antibodies to other flaviviruses that may circulate in the region such as Japanese encephalitis as a result of natural infection or vaccination may also affect the sensitivity of NS1 and should be considered. The data presented here are consistent with previous studies that report a higher sensitivity of the NS1 test in DENV-3 and DENV-1 infections compared to DENV-2 and DENV-4 infections [12,14]. As the specimens used in this study included all DENV serotypes, the overall sensitivity was not affected by a predominating serotype as has been reported elsewhere [7].

Our study also demonstrated that in secondary cases, the sensitivity is better in patients with more severe illness. The sensitivity of the test in the DF group was significantly lower compared to the other groups (DF+HM or DHF). DSS cases were excluded from this analysis, as the number of DSS cases was too small

(n=2). The association between NS1 and clinical severity has been studied before with discordant results [4,6,7,15,21]. The differences might be due to inadequate number of cases [21] or the application of different clinical category guidelines [4]. NS1 sensitivity was also significantly higher in patients with platelet counts below 100,000/$mm^3$ measured at presentation only or nadir platelet counts below 100,000/$mm^3$ if serial measurements were done. A positive NS1 result in secondary infections may therefore be a predictor for low platelet count. Although not significantly different, sensitivity tended to be higher in cases with spontaneous bleeding, effusion and hemoconcentration. The association between NS1 and clinical severity is thought to be related to the cross-reactivity of anti-dengue NS1 antibodies with platelets and endothelial cells, and the role of NS1 in the occurrence of vascular leakage through complement and cytokine responses [33,34]. Recent findings have also shown that NS1 positivity is correlated with higher viremia or antigenemia which was found in more severe cases [13,15]. The overall sensitivity of the NS1 test and its sensitivity in secondary infections in our study is lower than what has previously been published. There are several possible reasons for this difference. This and other studies have demonstrated that the sensitivity of NS1 in secondary infections is much lower than in primary infections. As a consequence, the overall sensitivity will be affected by the proportion of primary or secondary infections in the sample set used. Compared to studies conducted in other endemic countries, our study has a low proportion of primary infections. Our study also included outpatients (36.4%). As has previously been reported and is found in this study, NS1 sensitivity is associated with disease severity. Therefore, outpatients in our study, which were mostly mild cases, may have contributed to the lower NS1 sensitivity. Additionally, we found that among secondary cases, post-secondary (≥2) cases (cases with pre-illness neutralizing antibodies to more than one serotype) had lower sensitivities than secondary cases with pre-illness neutralizing antibodies to only one serotype. Based upon the data from the 47 subjects that were tested for pre-illness neutralizing antibodies and the mean age of our volunteers, we suspect that many of the cases in our study were post-secondary infections. Finally, the proportion of secondary infections caused by DENV-2 and DENV-4, serotypes with lower sensitivities in our study, was higher than in other studies.

The strengths of this study are: 1) work was conducted in a setting where all dengue serotypes were equally distributed and the ratio between primary and secondary infections reflected the regional epidemiology. 2) accurate clinical outputs were available as patients were monitored closely. 3) this study provides a wealth of laboratory data to assess the correlation between NS1 detection and serotype distribution, and dengue immune status including serotype specific pre-existing immunity, which have not been reported elsewhere. This study has also several limitations. First, male participants were more predominant than females. However, despite fewer samples from females, the detection of NS1 was significantly higher in females than males. Second, ultrasonography was not performed on a subset of volunteers. As a consequence, several DF+HM cases might be more accurately included in the DHF group. However, the numbers are probably small as daily hemoconcentration was performed on all individuals. Third, PRNT was only conducted on pre-illness sera from 47 patients. Although, the results showed a strong association between sensitivity and the presence of neutralizing antibodies to DENV-2, further study is needed.



In conclusion, the NS1 test is not recommended as a standalone diagnostic test for dengue infection in regions where secondary infections predominate. However, as the sensitivity and specificity in acute specimens from primary infections is excellent, use of the test may prove of value to travelers returning from dengue endemic areas. Furthermore, as the sensitivity in secondary cases is associated with clinical severity, NS1 may be useful to predict severe disease. However, further studies with larger sample sizes and in other geographic regions are needed to confirm this association.

## ACKNOWLEDGMENTS

We thank the dengue research team at NAMRU-2, Jakarta, at Hasan Sadikin Hospital/Padjadjaran University and Immanuel Hospital and the factory clinic at PT Grandtex Indonesia, Naintex, and Bintang Agung. Competing interests: None declared. Ethical approval: These studies were approved by the Institutional Review Board of NAMRU-2 (N2.2006.0001, N2.2004.0010, N2.2004.0008) and at the National Institute of Health Research and Development, Ministry of

Health, Indonesia (KS.02.01.2.1.2776, KS.02.01.2.1.3461 and KS.02.01.2.1.2336) in compliance with all U.S. Federal Regulations governing the protection of human subjects. Disclaimer: The views expressed in this article are those of the author and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the U.S. Government. Some of the authors are military service members or U.S. Government employees. This work was prepared as part of their official duties. Title 17 U.S.C. §105 provides that 'Copyright protection under this title is not available for any work of the United States Government.' Title 17 U.S.C. §101 defines a US Government work as a work prepared by a military service member or employee of the U.S. Government as part of that person's official duties.

## References

1. WHO (2009) Dengue and dengue hemorrhagic fever.
2. Farrar J (2008) Clinical features of dengue. In: Halstead SB, editor Dengue London: Imperial College pp: 171-191.
3. Winkler G, Maxwell SE, Ruemmler C, Stollar V (1989) Newly synthesized dengue-2 virus nonstructural protein NS1 is a soluble protein but becomes partially hydrophobic and membrane-associated after dimerization. Virology 171: 302-305.
4. Osorio L, Ramirez M, Bonelo A, Villar LA, Parra B (2010) Comparison of the diagnostic accuracy of commercial NS1-based diagnostic tests for early dengue infection. Virol J 7: 361.
5. Alcon S, Talarmin A, Debruyne M, Falconar A, Deubel V, et al. (2002) Enzyme-linked immunosorbent assay specific to Dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections. J Clin Microbiol 40: 376-381.
6. Libraty DH, Young PR, Pickering D, Endy TP, Kalayanarooj S, et al. (2002) High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever. J Infect Dis 186: 1165-1168.
7. Guzman MG, Jaenisch T, Gaczkowski R, Ty Hang VT, Sekaran SD, et al. (2010) Multi-country evaluation of the sensitivity and specificity of two commercially-available NS1 ELISA assays for dengue diagnosis. PLoS Negl Trop Dis 4.
8. Duong V, Ly S, Lorn Try P, Tuiskunen A, Ong S, et al. (2011) Clinical and virological factors influencing the performance of a NS1 antigen-capture assay and potential use as a marker of dengue disease severity. PLoS Negl Trop Dis 5: e1244.
9. Lima Mda R, Nogueira RM, Schatzmayr HG, dos Santos FB (2010) Comparison of three commercially available dengue NS1 antigen capture assays for acute diagnosis of dengue in Brazil. PLoS Negl Trop Dis 4: e738.
10. Blacksell SD, Mammen MP, Jr., Thongpaseuth S, Gibbons RV, Jarman RG, et al. (2008) Evaluation of the Panbio dengue virus nonstructural 1 antigen detection and immunoglobulin M antibody enzyme-linked immunosorbent assays for the diagnosis of acute dengue infections in Laos. Diagn Microbiol Infect Dis 60: 43-49.
11. Kumarasamy V, Wahab AH, Chua SK, Hassan Z, Chem YK, et al. (2007) Evaluation of a commercial dengue NS1 antigen-capture ELISA for laboratory diagnosis of acute dengue virus infection. J Virol Methods 140: 75-79.
12. Dussart P, Petit L, Labeau B, Bremand L, Leduc A, et al. (2008) Evaluation of two new commercial tests for the diagnosis of acute dengue virus infection using NS1 antigen detection in human serum. PLoS Negl Trop Dis 2: e280.

13. Hang VT, Nguyet NM, Trung DT, Tricou V, Yoksan S, et al. (2009) Diagnostic accuracy of NS1 ELISA and lateral flow rapid tests for dengue sensitivity, specificity and relationship to viraemia and antibody responses. PLoS Negl Trop Dis 3: e360.
14. Bessoff K, Delorey M, Sun W, Hunsperger E (2008) Comparison of two commercially available dengue virus (DENV) NS1 capture enzyme-linked immunosorbent assays using a single clinical sample for diagnosis of acute DENV infection. Clin Vaccine Immunol 15: 1513-1518.
15. Thomas L, Najioullah F, Verlaeten O, Martial J, Brichler S, et al. (2010) Relationship between nonstructural protein 1 detection and plasma virus load in Dengue patients. Am J Trop Med Hyg 83: 696-699.
16. Tricou V, Vu HT, Quynh NV, Nguyen CV, Tran HT, et al. (2010) Comparison of two dengue NS1 rapid tests for sensitivity, specificity and relationship to viraemia and antibody responses. BMC Infect Dis 10: 142.
17. Koraka P, Burghoorn-Maas CP, Falconar A, Setiati TE, Djamiatun K, et al. (2003) Detection of immune-complex-dissociated nonstructural-1 antigen in patients with acute dengue virus infections. J Clin Microbiol 41: 4154-4159.
18. Kyle JL, Harris E (2008) Global spread and persistence of dengue. Annu Rev Microbiol 62: 71-92.
19. Najioullah F, Combet E, Paturel L, Martial J, Koulmann L, et al. (2011) Prospective evaluation of nonstructural 1 enzyme-linked immunosorbent assay and rapid immunochromatographic tests to detect dengue virus in patients with acute febrile illness. Diagn Microbiol Infect Dis 69: 172-178.
20. Chaterji S, Allen JC, Jr., Chow A, Leo YS, Ooi EE (2011) Evaluation of the NS1 rapid test and the WHO dengue classification schemes for use as bedside diagnosis of acute dengue fever in adults. Am J Trop Med Hyg 84: 224-228.
21. Lapphra K, Sangcharaswichai A, Chokephaibulkit K, Tiengrim S, Piriyakarnsakul W, et al. (2008) Evaluation of an NS1 antigen detection for diagnosis of acute dengue infection in patients with acute febrile illness. Diagn Microbiol Infect Dis 60: 387-391.
22. Beckett CG, Kosasih H, Faisal I, Nurhayati, Tan R, et al. (2005) Early detection of dengue infections using cluster sampling around index cases. Am J Trop Med Hyg 72: 777-782.
23. Kosasih H, Ibrahim IN, Wicaksana R, Alisjahbana B, Hoo Y, et al. (2011) Evidence of human hantavirus infection and zoonotic investigation of hantavirus prevalence in rodents in western Java, Indonesia. Vector Borne Zoonotic Dis 11: 709-713.
24. de Souza V, Fernandes S, Araujo E, Tateno A, Oliveira O, et al. (2004) Use of an Immunoglobulin G Avidity Test To Discriminate between Primary and Secondary Dengue Virus Infections. J Clin Microbiol 42: 1782-1784.

25. WHO (1999) Prevention and control of dengue and dengue hemorrhagic fever- a comprehensive guidelines. New Delhi: WHO Regional Office for South-east Asia
26. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV (1992) Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J Clin Microbiol 30: 545-551.
27. Clarke DH, Casals J (1958) Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Am J Trop Med Hyg 7: 561-573.
28. Morens DM, Halstead SB, Repik PM, Putvatana R, Raybourne N (1985) Simplified plaque reduction neutralization assay for dengue viruses by semimicro methods in BHK-21 cells: comparison of the BHK suspension test with standard plaque reduction neutralization. J Clin Microbiol 22: 250-254.
29. Shu PY, Yang CF, Kao JF, Su CL, Chang SF, et al. (2009) Application of the dengue virus NS1 antigen rapid test for on-site detection of imported dengue cases at airports. Clin Vaccine Immunol 16: 589-591.
30. Myers RM, Varkey MJ (1971) A note on sequential dengue infection, presumptive and proved, with report of an instance of a third proved attack in one individual. Indian J Med Res 59: 1231-1236.
31. Kosasih H, H. Y, Sudjana P, Alisjahbana B, Wuryadi S, et al. (2006) Report of four volunteers with primary, secondary and tertiary dengue infections during a prospective cohort study. Dengue Bulletin 30: 87-92.
32. Duyen HT, Ngoc TV, Ha do T, Hang VT, Kieu NT, et al. (2011) Kinetics of plasma viremia and soluble nonstructural protein 1 concentrations in dengue: differential effects according to serotype and immune status. J Infect Dis 203: 1292-1300.
33. Martina BE, Koraka P, Osterhaus AD (2009) Dengue virus pathogenesis: an integrated view. Clin Microbiol Rev 22: 564-581.
34. Falconar AK (1997) The dengue virus nonstructural-1 protein (NS1) generates antibodies to common epitopes on human blood clotting, integrin/adhesin proteins and binds to human endothelial cells: potential implications in haemorrhagic fever pathogenesis. Arch Virol 142: 897-916.

**Table S1.** NS1 Study Results

| Author (Year/Country) | N (Gold standard) | Overall sensitivity[1] | Immune status | | | |
|---|---|---|---|---|---|---|
| | | | 1° case | 2° case | DENV1 | DENV2 |
| Osorio (2010/Colombia) | 212 (isolation, RT-PCR, serology) | 70.8% | 83-90[2] (29.4) | 40-50[2] (70.6) | 80-90[2] (27.4) | 50-60[2] (20) |
| Libraty (2002/Thailand) | 32 (RT-PCR) | 81.3% | 0 | 81.3 (100) | 0 | 81.3 (100) |
| Guzman (2010/multiple) | 1284 (isolation, RT-PCR, serology) | 66% (34-76%) | ND | ND | 87 (32.3) | 63 (20) |
| Hang (2009/Vietnam) | 125 (RT-PCR or serology) | 83.2% | 95.8 (19) | 78.5 (74) | 98 (50.4) | 55 (20) |
| Najioullah (2011/ Martinique) | 264 (RT-PCR) | 61.2% | 85 (28.6) | 48 (71.4) | 0 | 61.2 (100) |
| Chaterji (2011/Singapore) | 154 (isolation, RT-PCR) | 80.5% | 94.7 (48.7) | 67.1 (51.3) | 79.6 (35.1) | 73.9 (29.9) |
| Thomas (2010/Martinique) | 67 (RT-PCR) | 67.1% | 69.2 (74.2) | 61.5 (25.7) | 0 | 61.9 (30) |
| Blacksell (2008/Laos) | 38 (RT-PCR, serology) | 63.2% | 75 (10.5) | 60.1 (89.5) | 77.8 (23.7) | 60 (13.2) |
| Lima (2010/Brazil) | 220 (isolation, RT-PCR + serology) | 83.6% | 95 (74.1) | 71.4 (25.9) | 98 (22.7) | 90 (22.7) |
| Lapphra (2008/Thailand) | 171 (isolation, RT-PCR, serology) | 63.2% | 76.9 (7.6) | 64.5 (92.4) | NA | NA |
| Kumarasamy (2009/ Malaysia) | 213 (isolation, RT-PCR) | 93.4% | 97.3 (86.4) | 70 (13.6) | NA | NA |
| Bessoff (2008/Puerto Rico) | 208 (RT-PCR or isolation) | 83.2% | 98.3 (27.9) | 77.3 (72.1) | 92.9 (26.9) | 82.2 (21.6) |
| Tricou (2010/Vietnam) | 245 (RT-PCR) | 61.6% | 80.3 (27.3) | 55.1 (72.7) | NA (56.3) | NA (37.6) |
| Dussart (2008/French Guaina) | 272 (isolation, RT-PCR) | 87.4% (serotyped samples) | 96.3 (40.1) | 73 (59.9) | 90.9 (12.1) | 85.7 (15.4) |
| Duong (2011/Cambodia) | 243 (isolation, RT-PCR, NS1, serology) | 57.5% | 87.5 (14.5) | 53.5 (85.5) | 80 (21) | 60 (4) |

| Sensitivity[1] and proportion of specimens (both in %) by category | | | | | |
|---|---|---|---|---|---|
| Serotype | | | Day of specimen collection | Severity of Illness | Viremia (pfu/ml) |
| DENV3 | DENV4 | Unknown | | | |
| 80-90[2] (43.7) | 40-50[2] (8.9) | exact data not available | day 1-3:70-80[2] (NA), day 4-7 60-70[2] (NA) | **Non Severe:** 70-80[2] (75.7), **Severe:** 50-60[2] (24.3) | NA |
| 0 | 0 | 0 | NA | **DF:** 71(43.8), **DHF I,II,III:** 89(56.3) | associated with NS1 level which is higher in DHF than DF |
| 82 (11.1) | 79 (3.7) | 43.4 (37) | **Latin America[2]**, day2: 40-50, day3: 60-70, day 4: 60-80, day 5: ND, day 6: ND; **Southeast Asia[2],** day2: 70-80, day3: 70-80, day 4: 60-70, day 5: 50-60, day 6: 30-40 | **Latin America**: DF 41, DHF 68; **Southeast Asia**: DF: 70, DHF: 68 | NA |
| 96 (25) | ND (2.4) | ND (11.2) | day 0-3 90.6 (60), day 4-6 70 (40) | NA | Significantly higher in NS1 positive patients |
| 0 | 0 | 0 | day 1-5: 64.4 (NA), day 6: 37.9 (NA), day 7-8: 61.9 (NA) | NA | NA |
| 87 (35.1) | 0 | 0 | day 1: 64.1 (25.3), day 2: 82.8 (37.7), day 3: 89.5 (37), An increased sensitivity for secondary infection between days 0 and 3 | NA | NA |
| 0 | 69.4 (70) | 0 | day 1-3: 69.4 (70), day 4: 61.9 (30) | The proportion of NS1 was prolonged in secondary or severe infections | NS1+ correlated with viral loads |
| 0 (5.3) | 66.7 (23.7) | 61.5 (34.2) | day 1-4: 76.9 (16.5) day 5-7: 75 (83.5) | NA | NA |
| 86.2 (26.4) | 0 | 64.5 (28.2) | day 1-4: >80 (N/A), day 5-7: 75 (N/A) | NA | NA |
| NA | NA | N/A | Not associate with days of fever (63.2-75.7) | **DF:** 65.1 (88.3**);** **DHF:** 68.4 (11.7) | NA |
| NA | NA | 0 | NA | NA | NA |
| 86.5 (25) | 70.9 (26.4) | 0 | day 1: 70-80, day 2: 80-100, day 3: 70-80, day 4: 70-80, day 5: 80-100 | NA | Not related to NS1 positvity |
| NA (6.6) | 0 | 0 | ≤day 3: 60.9 (63.7), > day 3: 62.9 (36.3) | NA | NS1 + samples significantly higher viremia than NS1- |
| 87.1 (37.1) | 87 (16.9) | 60 (18.4) | day 0-3 89.7 (72.1) | NA | NA |
| 63.6 (46.5) | 53.3 (5.8) | 8.5 (22.7) | day 1-2 81 (8.8), day 3 60-80 (23), day 4 60-80(32.2), day 5 40-60 (20.1), day 6 20-40 (11.3), day 7-8 <20 (4.6) | **DF** 72.3 (39), **DHF/ DSS** 40.2 (35.8), **Indeterminate** NA (28) | NS1+ significantly higher in subjects with high viremia |

# Chapter 7

## Evidence for endemic chikungunya virus infections in Bandung, Indonesia

*PLoS Neglected Tropical diseases, 2013*

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## ABSTRACT

Chikungunya virus (CHIKV) is known to cause sporadic or explosive outbreaks. However, little is known about the endemic transmission of CHIKV. To ascertain the endemic occurrence of CHIKV transmission, we tested blood samples from patients with a non-dengue febrile illness who participated in a prospective cohort study of factory workers in Bandung, Indonesia. From August 2000 to June 2004, and September 2006 to April 2008, 1901 febrile episodes occurred and 231 (12.2%) dengue cases were identified. The remaining febrile cases were evaluated for possible CHIKV infection by measuring anti-CHIKV IgM and IgG antibodies in acute and convalescent samples. Acute samples of serologically positive cases were subsequently tested for the presence of CHIKV RNA by RT-PCR and/or virus isolation. A total of 135 (7.1%) CHIKV infections were identified, providing an incidence rate of 10.1/1,000 person years. CHIKV infections were identified all year round and tended to increase during the rainy season (January to March). Severe illness was not found and severe arthralgia was not a prominently reported symptom. Serial post-illness samples from nine cases were tested to obtain a kinetic picture of IgM and IgG anti-CHIKV antibodies. Anti-CHIKV IgM antibodies were persistently detected in high titers for approximately one year. Three patients demonstrated evidence of possible sequential CHIKV infections. The high incidence rate and continuous chikungunya cases in this adult cohort suggests that CHIKV is endemically transmitted in Bandung. Further characterization of the circulating strains and surveillance in larger areas are needed to better understand CHIKV epidemiology in Indonesia.

## SUMMARY

Chikungunya is one of the neglected diseases. It has only attracted attention during outbreaks, in particular, the large epidemics in the Indian Ocean in 2005-2006. To our knowledge, there has never been any surveillance to determine the transmission of this virus among humans in non-outbreak settings. Such surveillance is particularly important because it will provide a better estimate of the disease burden and valuable information on how this virus is maintained outside outbreaks. Our study, conducted between 2000 and 2008 in Bandung,

West Java, Indonesia, yielded several important findings. 1. Chikungunya is an important cause of fever among adults in Bandung, Indonesia. 2. The clinical symptoms are mostly mild and short lasting. 3. In addition to previously described epidemiological features involving episodic outbreaks, it is also continuously transmitted throughout the year. 4. A few patients may have experienced more than one chikungunya virus infection. 5. Only the Asian genotype was found and not the East Central South African genotype that was responsible for the 2005 outbreak in the Indian Ocean. 6. The persistence of IgM for a long period after illness may complicate the interpretation of laboratory results.

## INTRODUCTION

Chikungunya virus (CHIKV) is an arthropod-borne virus belonging to the genus *Alphavirus* in the family *Togaviridae* [1]. CHIKV causes an acute illness similar to dengue, characterized by fever, headache, nausea, vomiting, abdominal pain, myalgia, rash and arthralgia [2]. Arthralgia of the large joints may be severe and long lasting [3]. CHIKV was first identified in Tanzania in 1952 [4]. Over the next decades, it remained a relatively rare disease causing mostly small outbreaks in both Africa and Asia [2]. This changed dramatically after a mutation in the CHIKV E1 glycoprotein gene *(A226V)* occurred. This mutation enhanced the infectivity of the virus and its transmission by *Aedes albopictus* [5]. In 2005, the mutated CHIKV spread from the Indian Ocean where it produced large epidemics in India, Southeast Asia and Italy [6-9].

CHIKV in Africa is predominantly maintained in an inter-epidemic sylvatic cycle in which the virus resides in wild primates and mosquitoes such as *Aedes furcifer-taylori* and *Aedes africanus*. In Asia, *Aedes aegypti*, an anthrophophilic mosquito that lives in proximity with humans, has been the most significant vector [10]. In contrast, for the mutated CHIKV that caused the recent epidemic in the Indian Ocean, *Aedes albopictus* is the main vector [5].

In Indonesia, chikungunya was first reported in 1982 in East Sumatera. It then spread to other islands including Java, Kalimantan, Bali, Flores and Sulawesi [11]. After a hiatus of 15 years, sporadic outbreaks were reported simultaneously in several provinces on the island of Java in 2000-2002 [11].

Since then, clusters of cases have been reported sporadically from several provinces although the total number of cases reported has never exceeded 5,000 per year [12,13]. This number should be interpreted with caution, however, because similarities in symptoms between dengue and chikungunya [2] and logistic constraints in viral diagnostics in Indonesia [14] may have resulted in a gross underestimation of the incidence of chikungunya [15]. To better define the disease burden of chikungunya, active surveillance during non-outbreak periods is necessary. However, to our knowledge, no such studies have been conducted elsewhere. Therefore, to determine CHIKV transmission during inter-epidemic periods and the epidemiology of CHIKV infections in Indonesia, we analyzed the demographic, clinical and virological data collected from non-dengue acute febrile patients participating in a prospective adult cohort dengue study that was conducted in Bandung, West Java, Indonesia from 2000-2004 and 2006-2008.

## MATERIALS AND METHODS

### STUDY DESIGN

This study was a part of "An epidemiology study of dengue and dengue hemorrhagic fever in adults", approved by the Institutional Review Board of NAMRU#2, Jakarta (IRB#30855 and N2.2006.0001) and the National Institute of Health Research and Development (NIHRD), Ministry of Health, Indonesia (KS 02.02.2.1.2181, KS 02.01.2.1732 and KS.02.01.2.1.2776) in compliance with all U.S. Federal Regulations governing the protection of human subjects. Details of the study design are described elsewhere [16]. In brief, it was a textile factory-based prospective cohort study conducted in Bandung, West Java, Indonesia, a city that has more than 2 million inhabitants. The study was conducted in two phases, 2000-2004 and 2006 – 2008. Phase 1 was carried out in factories A and B, and phase 2 was carried out in factories A and C. A cohort of 2978 volunteers was maintained during the first phase and 2726 during the second phase with 44.5% of volunteers from cohort 1 also participating in cohort 2. All volunteers gave written informed consent prior to enrollment. During enrollment, demographic and health status data were obtained and baseline blood specimens were collected. On a quarterly basis, surveys were conducted, and blood samples

were taken to examine the volunteers' dengue serological status. Between each survey, volunteers who experienced fever came to the factory clinic where a clinical evaluation was performed and acute and convalescent (at least 7 days apart) blood specimens were collected. Specimens were immediately tested for dengue using a battery of dengue diagnostic assays [16]. Patients were advised to be hospitalized at the discretion of the attending physician or if their platelet count was less than 150,000/mm$^3$. Once a dengue infection was excluded, the samples were tested for evidence of CHIKV infection as described below. During phase 1 of the study, volunteers were not questioned about arthralgia and data concerning arthralgia was obtained when this symptom was a chief or other complaint during their illness or during post-illness serosurveys. During phase 2 of the study, volunteers were specifically asked about arthralgia. There were no other differences in symptom ascertainment between phase 1 and 2.

## DIAGNOSIS OF CHIKV INFECTIONS

Convalescent sera from febrile volunteers who had been excluded as dengue cases were first tested to detect CHIKV IgM antibodies using enzyme-linked immunosorbent assay (ELISA). When positive, paired acute and convalescent sera were further tested for CHIKV IgM and IgG antibodies using an ELISA, and acute sera were processed for viral isolation and/or tested by RT-PCR to detect CHIKV genomes. CHIKV infection was confirmed when CHIKV genome or virus was detected, or when either seroconversion or a four-fold rise in titers of anti-CHIKV IgM and IgG antibodies was detected.

### CHIKV IgM and IgG ELISA

Serum samples were assayed for the presence of IgG and IgM antibodies against CHIKV using ELISA as previously described [14,17]. The CHIK antigen was prepared from Vero E6 cell culture infected with CHIKV 23574, an Asian lineage virus isolated from CHIK infected patient. Uninfected Vero E6 cell culture was used as negative antigen. For detection of CHIK IgM, 96- well microtiter plates (Immulon 2, Dynex Technologies, Chantilly, VA) were coated with anti-human IgM antibodies (Kirkegaard and Perry, Gaithersburg, MD). Excess antibodies were

washed with 0.1% Tween Phosphate-buffered saline (PBS). Serum was diluted 1:100 in dilution buffer (PBS, 0.1% Tween-20, and 5% skim milk), and incubated at 37ºC for one hour. Plates were then washed and antigens were added. After incubation, anti-CHIK hyperimmune mouse ascitic fluid and horseradish peroxidase-conjugated anti-mouse IgG (Kirkegaard and Perry, Gaithersburg, MD) were used to detect IgM specific to CHIKV. ABTS substrate was allowed to react for one hour and absorbance was determined at 415 nm. For the detection of CHIK specific IgG antibodies, a 96-well microtiter plate was coated directly with cell lysate antigens diluted in PBS. Horseradish peroxidase conjugated mouse anti-human IgG Fc (Kirkegaard and Perry, Gaithersburg, MD), and ABTS were used to detect bound antibody. The adjusted optical density value (OD) for each sample was determined by subtracting the OD obtained with the negative antigen from the OD obtained using the CHIK antigen. A sample was considered positive if its OD value exceeded the mean plus three standard deviations of the normal control sera. The endpoint of antibody titers was determined by testing ELISA-positive samples at serial two-fold dilutions starting from 1:100. The highest dilution showing a positive result was considered the endpoint titer. It has previously been established that our CHIK immunoassay does not show immunoreactivity against Ross River virus [17].

The kinetics of IgM and IgG antibodies to CHIKV were analyzed using specimens from the first nine patients who had complete serial quarterly serosurvey sera for two years.

## Virus isolation and CHIKV RT-PCR

Acute sera from cases diagnosed by seroconversion or a four-fold increase of anti-CHIKV IgM and IgG antibody titers were processed for virus isolation and analysis by RT-PCR. The methods used to perform these assays have been described previously [14,17]. For virus isolation, serum samples were diluted 1:10 in PBS and applied to confluent monolayers of C6/36 cells in 24-well culture plates (Corning, New York). The plates were centrifuged at 400*g* for 45 minutes and then 1 ml of medium (MEM) added. The plates were then incubated at 30ºC for 14 days and observed daily for evidence of cytopathic effects (CPE). At the end

of 14 days or upon recognition of CPE, cells were removed from the plates and evaluated for the presence of virus by standard immunofluorescence assay using anti-CHIK hyperimmune mouse ascitic fluid and FITC conjugated anti-mouse IgG (Kirkegaard and Perry, Gaithersburg, MD). For RT-PCR, viral RNA was extracted using a QIAamp Viral RNA Isolation Kit (QIAGEN, Hilden, Germany). RNA was then used in a nested RT-PCR assay using JM1 (5' GCAGAC GCAGAGAGGGCCAG 3'; bp 1,201 to 1,220) and JM2 (5' CGTGCTGCAAGG TAGTTCTC 3'; bp 1,440 to 1,421) primers. A second nested PCR was performed using the product from the first reaction and primers JM3 (5' GCTATTTGTAAGAAC GTCAG 3'; bp 1,221 to 1,240) and JM4 (5'TACCGTGCTGCGGTCGGGAA 3'; bp 1,420-1,401). Amplified PCR products were resolved by electrophoresis on a 2% agarose gel and visualized using ethidium bromide.

## Genomic Sequencing

Sequencing of the structural polyprotein coding region of 20 chikungunya virus isolates was performed (Genbank accession numbers: KC879559-KC879578), using primers that were previously described [18]. Cycle sequencing reactions were conducted using the BigDye 3.3 Terminator Ready Reaction mix (Applied Biosystems, Carlsbad, California). Cycle sequencing was performed at least twice per primer per sample. The sequencing products were separated from unbound dye using BigDye X-Terminator (Applied Biosystems) and analyzed on an ABI 3130 XL Genetic Analyzer (Applied Biosystems).

Sequence data analysis was performed using Sequencher 3.1 (Genecodes, Ann Arbor, MI) with the default parameters to improve the overall sequence quality. ClustalX 2.0.9 [19] was used to perform multiple sequence alignment**.** The alignment parameters used were 50 points penalty for gap opening, 2 point penalty for gap extension, and all gaps were reset before each alignment. Several reference sequences were used for the alignment: six Asian genotype viruses (among them a vaccine strain), five East/Central/South African (ECSA) genotype viruses, and one West African genotype virus. After obtaining the alignment, excess sequence (outside the coding region for the structural proteins) was discarded. The phylogenetic tree was constructed using the neighbor-joining

method [20] with MEGA4 software [21]ur*C3. The distance model used was the Kimura 2 parameter model to correct for multiple substitutions and to account for unequal transition/transversion ratio. The tree was constructed with 1000 bootstrap replicates.

### Statistical analysis

Descriptive data (mean age and standard deviation) were analyzed using STATA version 9.0 (StataCorp 2005, College Station, TX).

## RESULTS

### Study Population

A total of 4380 volunteers were enrolled into the study, consisting of 1324 volunteers who joined from the beginning of this study (August 2000), 1654 who discontinued their participations after the first phase (June 2004) and 1402 new enrollees in the second phase (September 2006 to April 2008). The mean (SD) age and age range of volunteers at enrollment were 37.1 (±7.7) and 18 to 66 years old. A higher proportion of the study population was male (ratio 1.85: 1).

### Chikungunya Cases

#### *The proportion and incidence rate of chikungunya cases*

A total of 1431 acute febrile episodes (AFE) occurred among 2978 volunteers in the first phase of the study, which lasted for 47 months. CHIKV infection was identified in 96 (6.7%) of these AFE, yielding a yearly incidence rate of 10.1 per 1,000 persons. During the second phase of the study in which 2726 volunteers were followed for 20 months, 39 chikungunya cases were diagnosed among 470 AFE, resulting in a slightly higher percentage and incidence rate (8.3% and 10.3/1,000 persons/year). In total, the percentage and incidence rate were 7.1% and 10.1/1,000 (persons/year), respectively. The number of chikungunya cases per month over the years and the total number of cases per year are shown in figure 1. The number of cases remained relatively stable over the years, except for two peaks in early 2004 and 2008, when chikungunya accounted for 11.2% and

11.7% of the AFE. In contrast, no cases were found in the second half of 2000 and only one case in the last four months of 2006. Cases were found almost all year round, however November had the lowest number of cases and cases tended to increase during the wet season from January to March. Chikungunya cases resided throughout the city without apparent clustering by residence although the majority of cases were from the subdistricts near the factories where most workers lived (data not shown).

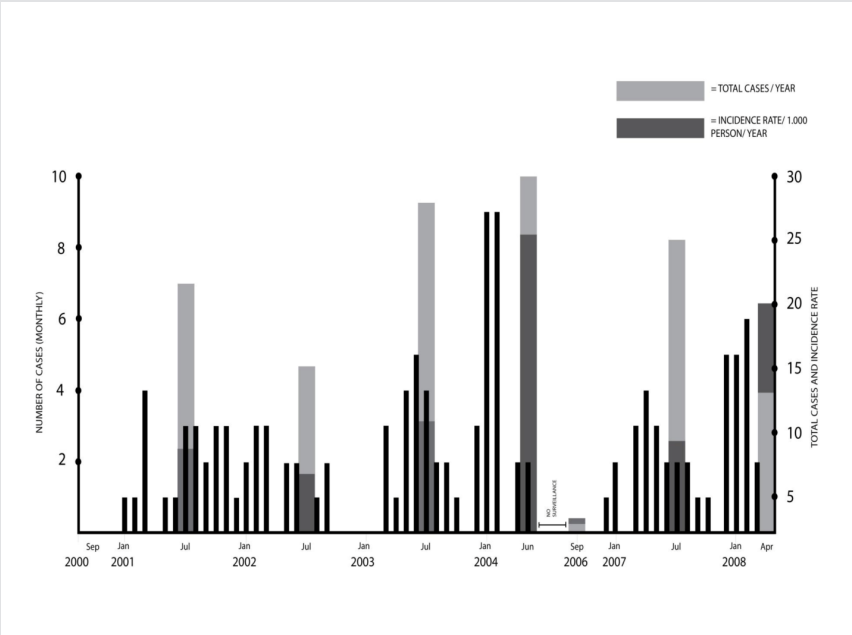


**Figure 1.** Monthly, annual total number and annual incidence rates for laboratory confirmed chikungunya (CHIK) cases.

The number of CHIK cases per month is represented by skinny black rectangles. The total number of cases for a year is indicated by light gray rectangles and the incidence rate for a year is indicated by dark gray rectangles. The rectangles representing annual data are placed at the midpoint (July) for their respective year.

### *Diagnosis of CHIKV infections, antibody kinetics and virus sequencing*

In 69 (51.1%) of the 135 CHIKV infections, the diagnosis of acute CHIKV infection was confirmed by positive RT-PCR, viral isolation and serology results. In 47 (34.8%) cases the diagnosis was confirmed by positive RT-PCR and serology results, while 19 (14.1%) cases only had serological evidence of CHIK infection. Of these 19 cases, 10 were negative for RT-PCR and isolation, eight did not have RT-PCR performed and one did not have viral isolation performed due to an insufficient volume of serum.

Post-illness sera from nine patients, who had serial specimens taken during serosurveys over two years, were tested to evaluate the kinetics of CHIKV IgM and IgG antibodies (figure 2A & 2B). These nine patients did not have any anti-

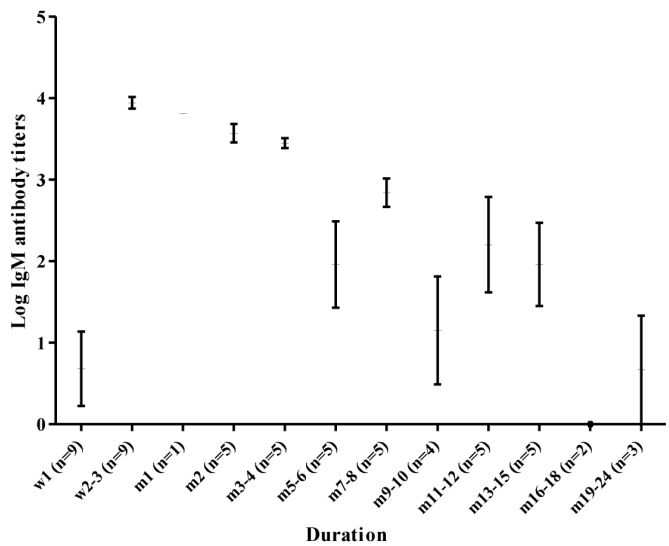


**Figure 2A.** Immunokinetics of anti-chikungunya virus IgM

The mean +/- SD titer for each timepoint (W=weeks, M=months) after illness onset. N = the number of samples for a given timepoint.

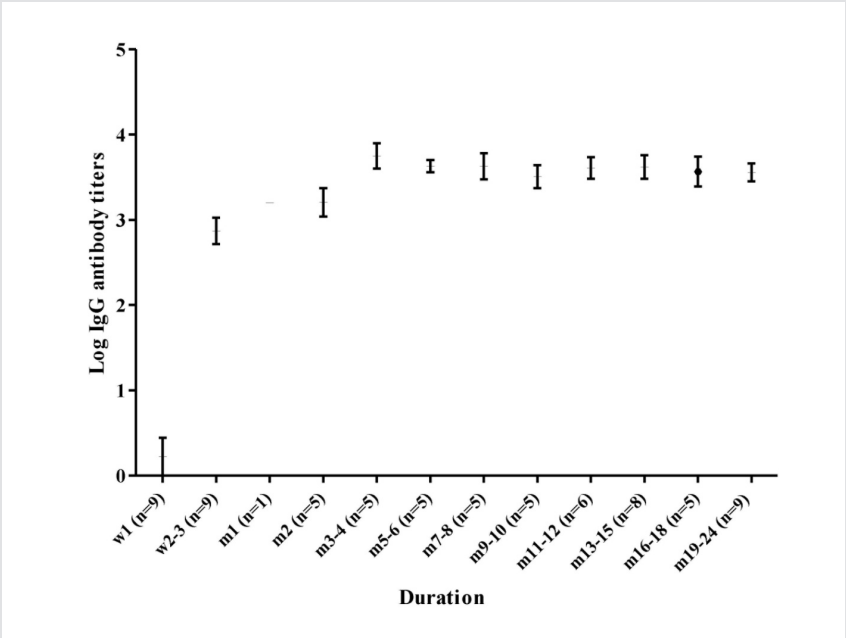


**Figure 2B.** Immunokinetics of anti-chikungunya virus IgG

The mean +/- SD titer for each timepoint (W=weeks, M=months) after illness onset. N = the number of samples for a given timepoint.

CHIKV IgM or IgG antibodies in their pre-illness samples and IgM antibodies were undetectable in the acute specimen of seven of these patients. The two patients in which IgM was detectable in the acute sample came to the clinic later in disease on day five and seven of illness and had anti-CHIKV IgM titers of 400 and 3200 respectively.

In convalescent and late convalescent sera, titers increased strongly, followed by a subsequent slow decline thereafter. In most patients (8/9), IgM antibodies were detectable for a long period after acute infection, ranging from 5-22 months. In one patient, the IgM titer became undetectable three months after the onset of illness. The demographic and clinical findings of this patient did not differ from the rest. IgG antibodies were not detected in 8 of 9 acute samples while

one sample had a titer of 100. In convalescent sera, which were collected 2-3 weeks after onset of fever, IgG antibodies were detected at low titers (200-800) in 7 of 9 patients and at high titers (1600 and 3200) in two patients. IgG titers peaked (6400 to 1:25600 dilution) at 3-4 months after the onset of illness, then decreased slightly and remained stable at high titers (1600 to 3200) for two years after illness.

CHIKV could be isolated from specimens collected until day 4 of the illness whereas RNA was detectable by RT-PCR until 6 days post illness onset. Sequencing analysis was performed on 20 isolates from the first and second phases of the study. All isolates had alanine at position 226 in the E1 gene. Nucleotide similarity between these isolates was >99.4%, and amino acid similarity was >99.7%. A phylogenetic tree was constructed based on 1320 bases of the structural polyprotein coding region. All samples sequenced from this study clustered together and belong to the Asian genotype (figure 3).

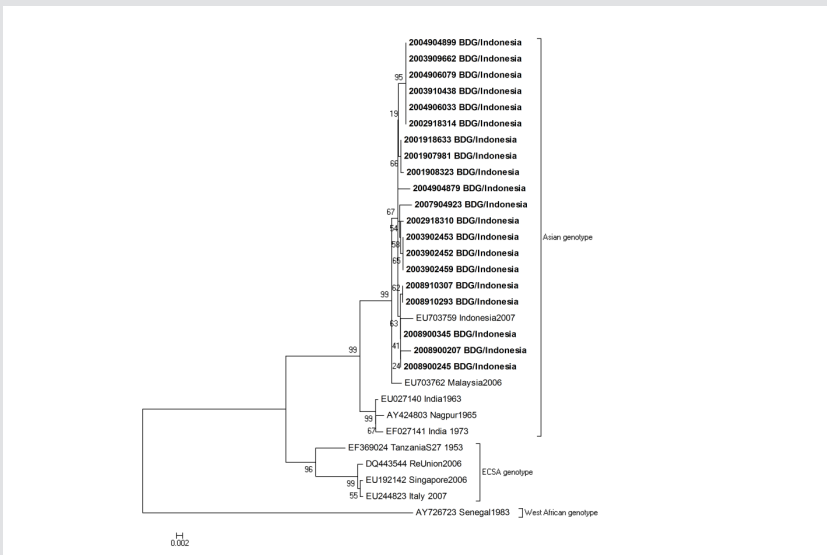


**Figure 3:** Phylogenetic analysis of the CHIKV isolates.

Neighbor-joining tree of the structural polyprotein coding regionof CHIKV. The sequences obtained in this study are highlighted in bold.Numbers indicate bootstrap values for the groups to the right.

### *Clinical manifestations*

Most patients came to the clinic on day two or three of fever (57 and 56 patients out of 135 patients, respectively). Table 1 lists their clinical manifestations.

**Table 1.** Signs, Symptoms and Laboratory Results for CHIK cases

| Symptoms | N | % Pos |
|---|---|---|
| Myalgia | 124/134 | 92.5 |
| Headache | 119/134 | 88.8 |
| Arthralgia | | |
| 1st phase | 37/95 | 38.9 |
| 2nd phase | 34/39 | 87.2 |
| Nausea | 71/134 | 53.0 |
| Retro-orbital pain | 51/134 | 38.1 |
| Cough | 37/134 | 27.6 |
| Abdominal Pain | 33/134 | 24.6 |
| Sore throat | 31/134 | 23.1 |
| Coryza | 31/134 | 23.1 |
| Rash | 17/134 | 12.7 |
| Vomiting | 15/134 | 11.2 |
| Diarrhea | 14/134 | 10.4 |
| Leukopenia(<4000/$mm^3$) | 20/134 | 14.9 |
| Thrombocytopenia (<150,000/$mm^3$) | 15/134 | 11.2 |

The most frequent were myalgia (92.5%), headache (88.8%), and arthralgia (38.9% during the first phase and 87.2%, during the second phase). Among 34 patients with arthralgia in the second phase of the study, the most commonly involved joints were the knees (85.3%), shoulders (64.7%), smaller joints and elbow (58.8%). The initial working diagnoses made by clinicians during enrollment at the health centers in cases with laboratory confirmed chikungunya included undifferentiated fever (71.1%), dengue fever (12.6%), upper respiratory tract infection (8.1%), typhoid fever (3%), chikungunya fever (1.5%), measles and

gastroenteritis (each 0.7%). Clinical manifestations were mostly mild and no severe cases occurred. Seven patients (5.2%) were hospitalized because their platelet level was under 150,000/mm$^3$, five from the first cohort and two from the second cohort. As chikungunya diagnostic assays were not available at the hospitals, discharged clinical diagnoses were acute viral infection and dengue fever in 5 and 2 patients, respectively. Approximately one third (32.6%) of patients did not skip work at all, 36.3% were absent for 1 to 3 days, 19.3% for 4 to 6 days and 11.9% for more than a week. The average number of days absent was 2.7 days.

## Evidence of possible recurrent CHIKV infections

We identified two patients with a laboratory confirmed acute CHIKV infection in whom serology results on a blood sample collected three months before illness suggested a previous CHIKV infection. In the first volunteer, an acute CHIKV infection was confirmed by RT-PCR, virus isolation and serology. In the second patient, CHIKV infection was confirmed by RT-PCR and serology (Table 2).

**Table 2.** Lab results for possible recurrent CHIKV infections

| ID Number | First infection | | Second infection | |
|---|---|---|---|---|
| | Date of illness | Lab results | Date of illness | Lab results |
| 005-1411 | unknown | Pre-illness specimen (21 SEP 2002) CHIKV IgM: 400 CHIK IgG: 800 | 4 JAN2003 | Positive RT-PCR and Isolation CHIKV IgM: 800 to 400 CHIKV IgG: 6400 to 6400 |
| 005-2048 | unknown | Pre-illness specimen (25 NOV2000) CHIKV IgM: 400 CHIK IgG: 800 | 6 MAR2001 | Positive RT-PCR CHIKV IgM: 100 to 400 CHIKV IgG: 3200 to 6400 |
| 005-1449 | 11 JUN 2002 | Positive RT-PCR and Isolation CHIKV IgM: neg to 6400 CHIKV IgG: neg to 100 | 19 DEC2006 | Negative RT-PCR and Isolation CHIKV IgM: 400-1600 CHIKV IgG: 1600-6400 |

Both patients reported fever, headache and myalgia, but no arthralgia. We also identified a 34-year-old male for whom we had evidence of two possible chikungunya episodes during our study. The first episode was in June 2002 when an acute CHIKV infection was confirmed by positive RT-PCR, virus isolation and IgM and IgG sero-conversion; the second episode occurred four and half years later and was confirmed by a four-fold rise in IgM (Table 2). For both episodes, dengue was excluded as all dengue diagnostic tests were negative. Clinical manifestations in both episodes were similar, including high fever, headache, sore throat, malaise and bilateral arthralgia. Results from hematology and chemistry tests did not show pathognomonic findings.

## DISCUSSION

Our studies, conducted between 2000 and 2008 in a large town in West Java, Indonesia, revealed several important epidemiological findings, including: 1. Among adults, CHIKV was an important cause of acute febrile illness. 2. CHIKV infections did not occur in epidemics as commonly reported, but were found throughout the year. 3. The clinical symptoms of CHIKV infection in this cohort were mostly mild and short-lived. 4. CHIKV infections were caused by the Asian genotype and not by the mutated East Central South African strain (ECSA), although only a limited number of samples were genotyped. 5. The persistence of IgM for a long period after illness may complicate the interpretation of laboratory results, and finally 6. We found evidence of possible recurrent CHIKV infections. We have previously reported an incidence rate of acute dengue of 18.1 cases per 1,000 persons per year (15.9% of febrile episodes) in the same cohort in 2000 to 2002 [16] and our present findings show that the corresponding incidence rate of chikungunya in these years was 7.9 cases per 1,000 persons per year (6.9% of febrile episodes). In 2004 and 2008, the incidence rates for chikungunya were in the same range as those for dengue (data not shown).The overall prevalence of CHIK between August 2000 to June 2004 and September 2006 to April 2008 was 7.1% of febrile episodes, and the overall incidence rate during this time frame was 10.1 cases per 1,000 persons per year.

Another finding of our study was that chikungunya infections were generally mild and of short duration. Only two-thirds of cases requested medical leave from work, with most only requesting 2-3 days of leave. The percentage of chikungunya cases in our study that were hospitalized was significantly lower than for the dengue cases detected in our study (unpublished data). Additionally, during the first phase of our study, when data on arthralgia was not specifically asked, only 38.9% of volunteers reported it as a chief or other complaint, suggesting this symptom was minor or absent. Also, we did not find volunteers with prolonged illness or complications. Similar to our clinical findings, mild chikungunya cases were also reported among young migrant workers in Singapore during a 2008 outbreak [22]. This is in contrast to what was reported in previous outbreaks where debilitating arthralgia was a frequent symptom in acute disease, sometimes persisting for months to even years [23-25] and in the recent La Reunion and India outbreaks where severe disease with neurological involvement was also reported [26,27].

Genotyping of the CHIKV in twenty patients showed that infections were caused by the Asian genotype and not the ECSA genotype, which was responsible for the 2005 outbreak in the Indian Ocean and has since then spread to India and Southeast Asia, causing unprecedented nationwide outbreaks in Malaysia, Singapore and Thailand [7]. The detection of the ECSA genotype has not been reported in Indonesia and the Asian genotype was the only genotype identified in Taiwan travelers returning from Indonesia in 2007-2008 [28] and in hospitalized patients in Surabaya in 2011 [15]. As ECSA genotype has been detected elsewhere in Southeast Asia [18,29-31], this genotype may have circulated in Indonesia as well, but remains unidentified as routine chikungunya surveillance has not been established. Still little is known about differences in clinical presentation and epidemiology between infections caused by the Asian and the ECSA genotype. Our findings suggest that the illness caused by this Asian genotype is often relatively mild and that infections occur year round in Bandung, Indonesia. Interestingly, infections by the same Asian genotype that were reported in travelers returning from Indonesia [32] and during outbreaks in Indonesia [11,17] appear to be associated with more severe disease. Selection bias, whereby chikungunya was only considered in those with more severe disease, and differences in the CHIKV strains that circulate during the inter-

epidemic period and those causing outbreaks and/or severe illness might explain these differences. Another plausible explanation of mostly mild cases in our study was the young adult population in contrast to children or the elderly who commonly experience severe illness [33-35].

According to our literature review, CHIKV infections have commonly been associated with outbreaks [7] and many countries reported a long hiatus between outbreaks, for example 32 years in India, 15 years in Indonesia and 7 years in Malaysia [11,36,37]. To the best of our knowledge, our study is the first to report year round CHIKV infections over several years. As such, it provides a better understanding of how CHIKV is maintained in the population. It was speculated that unreported infections were also the cause of an outbreak in Malaysia in 2006 [36]. One of the reasons chikungunya is not reported is the difficulty in distinguishing chikungunya clinically from other infections, such as dengue [2,38]. This may be especially challenging when arthralgia, which is considered a pathognomonic symptom of chikungunya, is not a prominent clinical feature. Moreover, chikungunya is generally perceived to occur only in outbreaks [39]. The limited sensitivity of currently available rapid diagnostic tests for chikungunya and the long persistence of CHIKV IgM antibodies may further complicate the correct diagnosis of chikungunya [14,40]. Advanced diagnostic tools such as virus isolation and RT-PCR are generally only available in large hospitals or research institutions. In our study, chikungunya cases were identified almost every month throughout the year from 2001-2004 and 2006-2008. The peak of chikungunya cases was during or after the monsoon season when the population of *Aedes aegypti* is abundant. This finding is consistent with previous reports from other countries [41-43]. In addition, acute chikungunya infections were still detected, albeit at a lower frequency, during the dry season, suggesting that virus transmission was maintained during this period. This is not surprising as *Aedes aegypti* and *albopictus* were found abundantly in Bandung and elsewhere in Indonesia throughout the year despite extensive eradication efforts [11,44-47]. Based on the data from this study, we did not observe any clustering or focused geographical transmission (data not shown).

One of the powerful features of our prospective cohort study was that volunteers were followed for several years. Therefore, we were able to observe the kinetics

of CHIKV IgM and IgG antibodies longitudinally after infection. Our finding that IgM antibodies could be detected beyond one year was consistent with previous reports [41,48]. In a returning traveler from La Reunion Island, IgM antibodies remained detectable after two years and this was associated with persistent arthralgia [48]. In our study, among nine patients observed, IgM antibodies disappeared between five months and 19 months (median of 10 months) after infection. Post-illness sera were collected during programmed serosurveys and not related to any febrile episodes, arguing against the possibility that the high titers of anti-CHIKV antibodies were induced by a nonspecific polyclonal activation after unrelated infections.

Persistence of IgM antibodies also has consequences for diagnostics. In the absence of virus culture or RT-PCR, IgM and IgG serology assays should be conducted using paired sera collected at least 10 days apart to confirm the increasing titers. We also identified three patients (2%) with symptomatic CHIKV infections with laboratory features suggestive of possible secondary chikungunya infection, which, to our knowledge, has not yet been reported. Indeed, the current dogma holds that CHIKV infection will provide life-long immunity [49]. In one of these three patients, the RT-PCR for CHIKV was negative and the diagnosis of an acute chikungunya infection was based on a rapid increase in both IgM and IgG. This rapid increase in IgM and IgG, which also may have resulted in rapid virus clearance, was different from the kinetics of IgM and IgG antibodies during primary infections. IgM antibodies in primary infections were often not detected in acute sera or only identified in low titers, followed by a rapid increase during convalescence, when IgG antibodies first became detectable. We could not perform sequence analysis to identify the differences between CHIKV isolates in primary and secondary infections because, in two cases, the first infections occurred prior to their participation in this study while in the third case, CHIKV could be isolated only from the first infection. Cross-reactivity with viruses belonging to the Semliki group is a potential explanation. However, Ross River and O'nyong nyong viruses have never been reported in Indonesia and our CHIKV IgM immunoassay does not show immunoreactivity with the Ross River virus [17].

In the absence of virological data from human cases or mosquitoes due to the scarcity of chikungunya research in Indonesia, we speculate that these three patients in Bandung were re-infected by different CHIKV strains. Further studies are needed to confirm this hypothesis, including close longitudinal observation of those who experienced first CHIKV infections and performance of plaque reduction neutralization tests, which were not available in our laboratory due to the BSL-3 containment requirement.

Our study has some limitations. First, data on arthralgia as a symptom during the first phase of the study was collected based on passive reports from patients during their visits. On the other hand, it may also provide us some information regarding the percentage of patients who consider arthralgia as a prominent symptom. Listing arthralgia as one of the subjective symptoms that should be routinely included in questionnaires during acute illness may unintentionally increase the likelihood of subjects endorsing this symptom. Second, as mentioned above, the diagnosis of previous infections in two cases and recurrent infection in one case was based solely on results obtained by ELISA. Detecting the presence of virus either by tissue culture isolation or RT-PCR, or performing plaque reduction neutralization assays on the serum samples would have provided more definitive evidence for recurrent infections.

In conclusion, our findings provide an estimate of the disease burden of CHIKV infections in Bandung, Indonesia and especially provide new information on its endemic transmission during inter-epidemic periods. These data highlight the importance of considering chikungunya in the differential diagnoses of acute febrile illnesses. Further studies are required to determine the significance of persistent IgM antibodies and the relation to arthralgia, the possibility of repeat CHIKV infections and the differences between strains in their potential to cause severe illness and epidemic versus endemic transmission. In addition, national surveillance needs to be established to monitor for the possible introduction of the ECSA genotype into Indonesia, as the transmission of this genotype may have a greater impact on public health. Finally, our findings highlight the need for development of affordable and sensitive rapid antigen diagnostic tests for early diagnosis of CHIKV infections and the need for a vaccine, especially since vector control has been unsuccessful so far.

## ACKNOWLEDGEMENTS

We would like to thank all volunteers for their valuable working hours, Dr Ardini Raksanegara and Ms Hodijah at the factory clinics, research teams from the Faculty of Medicine, Universitas Padjadjaran, Hasan Sadikin Hospital and NAMRU#2 for their dedication to this study, Wibowo Arindrarto for his work on chikungunya sequencing of 2001-2004 isolates, Kendra Chittenden from USAID Indonesia and Indonesia Research Partnership on Infectious Diseases (INA-RESPOND) for their support during the preparation of this manuscript.

## References


1. Schwartz O, Albert ML (2010) Biology and pathogenesis of chikungunya virus. Nat Rev Microbiol 8: 491-500.
2. Burt FJ, Rolph MS, Rulli NE, Mahalingam S, Heise MT (2012) Chikungunya: a re-emerging virus. Lancet 379: 662-671.
3. Borgherini G, Poubeau P, Jossaume A, Gouix A, Cotte L, et al. (2008) Persistent Arthralgia Associated with Chikungunya Virus: A Study of 88 Adult Patients on Reunion Island. Clinical Infectious Diseases 47: 469-475.
4. Robinson M (1955) An epidemic of virus disease in Southern Province, Tanganyika Territory, in 1952–53. I. Clinical features. Trans R Soc Trop Med Hyg 49: 28–32.
5. Tsetsarkin KA, Vanlandingham DL, McGee CE, Higgs S (2007) A Single Mutation in Chikungunya Virus Affects Vector Specificity and Epidemic Potential. PLoS Pathog 3: e201.
6. Kumar NP, Joseph R, Kamaraj T, Jambulingam P (2008) A226V mutation in virus during the 2007 chikungunya outbreak in Kerala, India. J Gen Virol 89: 1945-1948.
7. Pulmanausahakul R, Roytrakul S, Auewarakul P, Smith DR (2011) Chikungunya in Southeast Asia: understanding the emergence and finding solutions. International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases 15: e671-e676.
8. Yap G, Pok KY, Lai YL, Hapuarachchi HC, Chow A, et al. (2010) Evaluation of Chikungunya diagnostic assays: differences in sensitivity of serology assays in two independent outbreaks. PLoS Negl Trop Dis 4: e753.
9. Rezza G, Nicoletti L, Angelini R, Romi R, Finarelli AC, et al. (2007) Infection with chikungunya virus in Italy: an outbreak in a temperate region. The Lancet 370: 1840-1846.
10. Vazeille M, Moutailler S, Pages F, Jarjaval F, Failloux AB (2008) Introduction of Aedes albopictus in Gabon: what consequences for dengue and chikungunya transmission? Trop Med Int Health 13: 1176-1179.
11. Laras K, Sukri NC, Larasati RP, Bangs MJ, Kosim R, et al. (2005) Tracking the re-emergence of epidemic chikungunya virus in Indonesia. Trans R Soc Trop Med Hyg 99: 128-141.
12. Pusdatin (2009) Profil Kesehatan Indonesia 2008. Departemen Kesehatan Republik Indonesia: 47-48.
13. Pusdatin (2008) Profil Kesehatan Indonesia 2007. Departemen Kesehatan Republik Indonesia: 45.
14. Kosasih H, Widjaja S, Surya E, Hadiwijaya S, Butarbutar DPR, et al. (2012) Evaluation

of two IgM rapid immunochromatographic tests during circulation of Asian lineage Chikungunya virus. Southeast Asian Journal of Tropical Medicine and Public Health 43: 55-61.

15. Mulyatno KC, Susilowati H, Yamanaka A, Soegijanto S, Konishi E (2012) Primary isolation and phylogenetic studies of Chikungunya virus from Surabaya, Indonesia. Jpn J Infect Dis 65: 92-94.
16. Porter KR, Beckett CG, Kosasih H, Tan RI, Alisjahbana B, et al. (2005) Epidemiology of Dengue and Dengue Hemorrhagic Fever in a Cohort of Adults Living in Bandung, West Java, Indonesia. 72: 60-66.
17. Porter KR, Tan R, Istary Y, Suharyono W, Sutaryo, et al. (2004) A serological study of Chikungunya virus transmission in Yogyakarta, Indonesia: evidence for the first outbreak since 1982. Southeast Asian J Trop Med Public Health 35: 408-415.
18. Ng LC, Tan LK, Tan CH, Tan SS, Hapuarachchi HC, et al. (2009) Entomologic and virologic investigation of Chikungunya, Singapore. Emerg Infect Dis 15: 1243-1249.
19. Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, et al. (2003) Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res 31: 3497-3500.
20. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406-425.
21. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24: 1596-1599.
22. Win MK, Chow A, Dimatatac F, Go CJ, Leo YS (2010) Chikungunya fever in Singapore: acute clinical and laboratory features, and factors associated with persistent arthralgia. J Clin Virol 49: 111-114.
23. Borgherini G, Poubeau P, Jossaume A, Gouix A, Cotte L, et al. (2008) Persistent arthralgia associated with chikungunya virus: a study of 88 adult patients on reunion island. Clin Infect Dis 47: 469-475.
24. Larrieu S, Pouderoux N, Pistone T, Filleul L, Receveur MC, et al. (2010) Factors associated with persistence of arthralgia among Chikungunya virus-infected travellers: report of 42 French cases. J Clin Virol 47: 85-88.
25. Chopra A, Anuradha V, Ghorpade R, Saluja M (2012) Acute Chikungunya and persistent musculoskeletal pain following the 2006 Indian epidemic: a 2-year prospective rural community study. Epidemiol Infect 140: 842-850.
26. Chandak NH, Kashyap RS, Kabra D, Karandikar P, Saha SS, et al. (2009) Neurological complications of Chikungunya virus infection. Neurol India 57: 177-180.
27. Tournebize P, Charlin C, Lagrange M (2009) [Neurological manifestations in Chikungunya: about 23 cases collected in Reunion Island]. Rev Neurol (Paris) 165: 48-51.

28. Huang JH, Yang CF, Su CL, Chang SF, Cheng CH, et al. (2009) Imported chikungunya virus strains, Taiwan, 2006-2009. Emerg Infect Dis 15: 1854-1856.
29. Duong V, Andries AC, Ngan C, Sok T, Richner B, et al. (2012) Reemergence of Chikungunya virus in Cambodia. Emerg Infect Dis 18: 2066-2069.
30. Sam IC, Chan YF, Chan SY, Loong SK, Chin HK, et al. (2009) Chikungunya virus of Asian and Central/East African genotypes in Malaysia. J Clin Virol 46: 180-183.
31. Theamboonlers A, Rianthavorn P, Praianantathavorn K, Wuttirattanakowit N, Poovorawan Y (2009) Clinical and molecular characterization of chikungunya virus in South Thailand. Jpn J Infect Dis 62: 303-305.
32. Mizuno Y, Kato Y, Takeshita N, Ujiie M, Kobayashi T, et al. (2011) Clinical and radiological features of imported chikungunya fever in Japan: a study of six cases at the National Center for Global Health and Medicine. Journal of Infection and Chemotherapy 17: 419-423.
33. Haas H, Robin S, Ramful D, Houdon L, Minodier P, et al. (2009) [Chikungunya virus infections in children]. Arch Pediatr 16 Suppl 2: S72-79.
34. Pellot AS, Alessandri JL, Robin S, Samperiz S, Attali T, et al. (2012) [Severe forms of chikungunya virus infection in a pediatric intensive care unit on Reunion Island]. Med Trop (Mars) 72 Spec No: 88-93.
35. Borgherini G, Poubeau P, Staikowsky F, Lory M, Le Moullec N, et al. (2007) Outbreak of chikungunya on Reunion Island: early clinical and laboratory features in 157 adult patients. Clin Infect Dis 44: 1401-1407.
36. AbuBakar S, Sam IC, Wong PF, MatRahim N, Hooi PS, et al. (2007) Reemergence of endemic Chikungunya, Malaysia. Emerg Infect Dis 13: 147-149.
37. Lahariya C, Pradhan S (2006) Emergence of chikungunya virus in Indian subcontinent after 32 years: A review. J Vector Borne Dis December, 43: 151-160.
38. Lee VJ, Chow A, Zheng X, Carrasco LR, Cook AR, et al. (2012) Simple Clinical and Laboratory Predictors of Chikungunya versus Dengue Infections in Adults. PLoS Negl Trop Dis 6: e1786.
39. Hertz JT, Munishi OM, Ooi EE, Howe S, Lim WY, et al. (2012) Chikungunya and Dengue Fever among Hospitalized Febrile Patients in Northern Tanzania. The American Journal of Tropical Medicine and Hygiene 86: 171-177.
40. Rianthavorn P, Wuttirattanakowit N, Prianantathavorn K, Limpaphayom N, Theamboonlers A, et al. (2010) Evaluation of a rapid assay for detection of IgM antibodies to chikungunya. Southeast Asian Journal of Tropical Medicine and Public Health 41: 92-96.
41. Ray P, Ratagiri VH, Kabra SK, Lodha R, Sharma S, et al. (2012) Chikungunya infection in India: results of a prospective hospital based multi-centric study. PLoS One 7: e30025.

42. Thaikruea L, Charearnsook O, Reanphumkarnkit S, Dissomboon P, Phonjan R, et al. (1997) Chikungunya in Thailand: a re-emerging disease? Southeast Asian J Trop Med Public Health 28: 359-364.
43. Gerardin P, Barau G, Michault A, Bintner M, Randrianaivo H, et al. (2008) Multidisciplinary prospective study of mother-to-child chikungunya virus infections on the island of La Reunion. PLoS Med 5: e60.
44. Mulyatno KC, Yamanaka A, Ngadino, Konishi E (2012) Resistance of Aedes aegypti (L.) larvae to temephos in Surabaya, Indonesia. Southeast Asian J Trop Med Public Health 43: 29-33.
45. Bangs MJ, Larasati RP, Corwin AL, Wuryadi S (2006) Climatic factors associated with epidemic dengue in Palembang, Indonesia: implications of short-term meteorological events on virus transmission. Southeast Asian J Trop Med Public Health 37: 1103-1116.
46. Akbar MR AR, Djatie T, Kodyat S (2008) PCR Detection of Dengue Transovarial Transmissibility in Aedes aegypti in Bandung, Indonesia. Proc ASEAN Congr Trop Med Parasitol 3: 6.
47. Ahmad I, Astari S, Tan M (2007) Resistance of Aedes aegypti (Diptera: Culicidae) in 2006 to pyrethroid insecticides in Indonesia and its association with oxidase and esterase levels. Pak J Biol Sci 10: 3688-3692.
48. Malvy D, Ezzedine K, Mamani-Matsuda M, Autran B, Tolou H, et al. (2009) Destructive arthritis in a patient with chikungunya virus infection with persistent specific IgM antibodies. BMC Infect Dis 9: 200.
49. Powers AM (2010) Chikungunya. Clin Lab Med 30: 209-219.

# Chapter 8

## Surveillance of influenza in Indonesia, 2003–2007

*Influenza and other respiratory viruses, 2013*

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## ABSTRACT

### BACKGROUND

Longitudinal data are limited about the circulating strains of influenza viruses and their public health impact in Indonesia. We conducted influenza surveillance among outpatients and hospitalized patients with influenza-like illness (ILI) across the Indonesian archipelago from 2003 through 2007.

### METHODOLOGY

Demographic, clinical data, and respiratory specimens were collected for 4,236 ILI patients tested for influenza virus infection by RT-PCR and viral culture.

### PRINCIPAL FINDINGS

Influenza A and B viruses co-circulated year round with seasonal peaks in influenza A virus activity during the rainy season (December–January). During 2003–2007, influenza viruses were identified in 20,1% (4,236/21,030) of ILI patients, including 20,1% (4,015/20,012) of outpatients, and 21,7% (221/1,018) of inpatients. One H5N1 case was identified retrospectively in an outpatient with ILI. Antigenic drift in circulating influenza A and B virus strains was detected during the surveillance period in Indonesia. In a few instances, antigenically drifted viruses similar to the World Health Organization (WHO) vaccine strains were detected earlier than the date of their designation by WHO.

### CONCLUSION

Influenza A and B virus infections are an important cause of influenza-like illness among outpatients and hospitalized patients in Indonesia. While year-round circulation of influenza viruses occurs, prevention and control strategies should be focused upon the seasonal peak during rainy season months. Ongoing virologic surveillance and influenza disease burden studies in Indonesia are important priorities to better understand the public health impact of influenza in South-East Asia and the implications of influenza viral evolution and global spread.

## INTRODUCTION

While the disease burden and seasonality of influenza virus activity in temperate regions of the Northern and Southern Hemispheres have been well characterized with clear peaks during winter months [1,2], such data are limited for developing countries with tropical and subtropical climates [3]. Recent studies suggest that South-East Asia is an important region in the global ecology and evolution of influenza viruses [4,5]. The ongoing epizootic of highly pathogenic avian influenza A (H5N1) virus among poultry with sporadic transmission to humans further highlights the importance of influenza virus surveillance and response measures in South-East Asia [6,7].

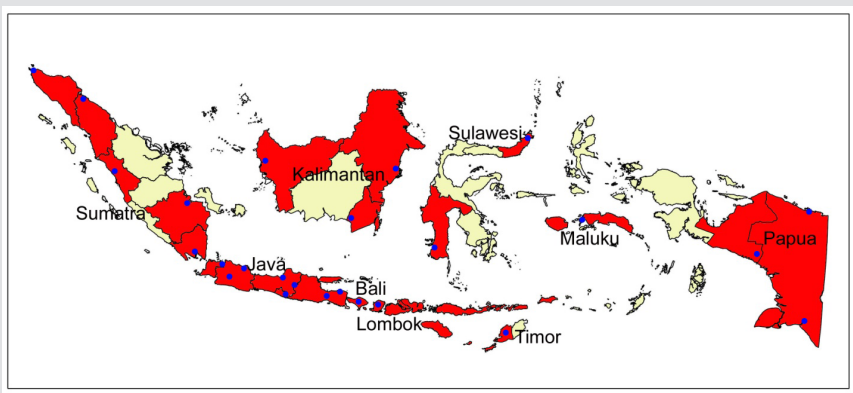
In tropical and subtropical developing countries, influenza is often under-diagnosed and the disease burden and clinical severity are under-estimated [3]. However, recent studies have revealed that influenza virus infections in tropical climate countries, including in South-East Asia, cause similar severity and disease burden as in temperate countries [8,9]. Beginning in 2003, a widespread, ongoing epizootic of highly pathogenic avian influenza A (H5N1) virus has impacted poultry in Indonesia. First detected in 2005, sporadic H5N1 virus transmission to humans has resulted in high mortality [7]. Since H5N1 virus infection can also cause severe influenza-like illness (ILI) among exposed persons, virologic surveillance is needed to understand the relative impact of H5N1 virus and seasonal influenza A and B viruses among persons with ILI in Indonesia.

Influenza surveillance was implemented among outpatients at six sentinel sites in three districts in Java, Indonesia between September 1999 and January 2003 [10]. In this report, we describe the findings of expanded influenza surveillance at additional sites across the Indonesian archipelago, including among hospitalized patients, during 2003–2007.

# METHODS

## SURVEILLANCE SITES

We conducted surveillance for influenza-like illness (ILI) among patients seeking care at primary health centers (outpatients) and hospitals (outpatients and inpatients) across Indonesia during January 2003 to December 2007. The number of participating facilities expanded from five sentinel sites in five districts in 2003 to 22 sites in 18 districts in 2004–2005 and 48 sites (28 outpatient sites at primary health care centers and hospitals, and 20 inpatient sites) in 26 districts in 2006–2007, covering 22 of 33 Indonesian provinces (Figure 1).




| | Suma-tera | Java | Kali-mantan | Bali | Lombok | Sulawesi | Maluku | Timor | Papua | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| Number of health facilities and (districts), by year | | | | | | | | | | |
| 2003 | 0 | 3(3) | 0 | 1(1) | 0 | 1(1) | 0 | 0 | 0 | 5(5) |
| 2004 – 2005 | 3(3) | 9(5) | 2(2) | 1(1) | 1(1) | 2(2) | 1(1) | 1(1) | 2(2) | 22(18) |
| 2006 – 2007 | 9(6) | 23(8) | 4(3) | 1(1) | 1(1) | 4(2) | 1(1) | 1(1) | 4(3) | 48(26) |
| Total population, million | | | | | | | | | | |
| | 50·7 | 136·6 | 13·8 | 3·9 | 2·6 | 17·4 | 2·6 | 1 | 3·6 | 232·2 |

**Figure 1.** Location of Indonesia influenza surveillance sites (blue) and provinces with surveillence sites (red), 2003–2007

## SURVEILLANCE METHODS

### Patients 'enrollment and specimen collection'

We enrolled patients who presented at primary health centers (outpatients) and hospitals (outpatients and inpatients) who met the inclusion criteria of ILI, defined as a measured axillary temperature of ≥37.8$^0$C or history of feverishness, and either cough or sore throat without any other diagnosis. Among patients meeting the ILI case definition, a convenience sample of up to 20 ILI cases per week was enrolled. Clinicians or trained nurses obtained demographic data and clinical symptoms, performed physical examinations, and collected nasal and throat swabs from enrolled ILI cases. Swab specimens were placed into sterile Hanks' balanced salt solution (HBSS) viral transport media (VTM) that contained gelatin, 100 µ/ml penicillin, 100 µg/ml, streptomycin, and 25 U/ml mycostatin. Specimens were refrigerated (4$^0$C) and shipped weekly to laboratories in Jakarta for testing Specimens from suspected H5N1 patients were shipped to laboratories in Jakarta within 24 hours of collection for urgent H5 testing [6].

### Laboratory evaluation

From 2003 to September 2005, all respiratory specimens were tested for influenza viral RNA by conventional reverse transcription polymerase chain reaction (RT-PCR) assay. The QIAamp Viral RNA kit (QIAGEN, Valencia, CA, USA) was used to extract viral RNA from prepared samples. Samples were assayed using multiplex nested reverse transcription RT-PCR (MnRT-PCR) to detect human influenza viral RNA. In the MnRT-PCR, viral RNA was amplified utilizing cocktails of oligonucleotide primers [11] directed collectively against the matrix protein (MP), hemagglutinin (HA), and neuraminidase (NA) genes for influenza A (H1N1) and A (H3N2) viruses, and the MP and HA genes for influenza B virus. Primers targeting H5 were not included in the nested MnRT-PCR. Amplicons were separated by electrophoresis on 2% agarose gel containing ethidium bromide for virus type and sub-type identification. Positive specimens were inoculated into Madin–Darby canine kidney (MDCK) tissue cells for viral isolation.

Beginning in October 2005, all specimens were first screened for influenza A

(H5) viral RNA using real-time RT-PCR (rRT-PCR) as described below. Negative H5 specimens were tested for influenza A (H1N1) (H3N2) and influenza B viral RNA using conventional RT-PCR as described above. Specimens testing positive for seasonal influenza A or B viruses were placed into MDCK tissue cells for viral isolation. Virus isolates were characterized by hemagglutination inhibition (HAI) assay as previously described [12]. All influenza virus testing was performed at the National Institute of Health Research and Development, Ministry of Health (NIHRD, MoH), Indonesia, and U.S. Naval Medical Research Unit #2 (NAMRU#2), Jakarta, utilizing the same standard operating procedures. A convenience sample of approximately 45% of isolates was sent for confirmation and antigenic characterization at the World Health Organization Influenza Collaborating Center (WHO-CC) at the U.S. Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, periodically until mid-2006.

## rRT-PCR assay

Starting in October 2005, all specimens were first screened for H5 by rRT-PCR. Ribonucleic acid (RNA) was extracted from nasal and throat swabs using QIAamp viral RNAmini kits (QIAGEN, Hilden, Germany) following the manufacturer's instruction and stored at -70$^0$C. For detection of A (H5) viral RNA, rRT-PCR was initially conducted using an H5 primer set, [13] and then later in 2006, using primers and probes designed by the CDC to specifically recognize the subclade 2.1 viruses circulating among poultry with sporadic transmission to humans in Indonesia. One-step rRTPCR was performed in a final volume of 25 µl containing 5 µl of extracted RNA, 12.5 µl of buffer mix and 0.5 µl Superscript III / Platinum Taq-Enzyme mix, 20 unit of RNase-out (Invitrogen, Carlsbad, CA, USA), 0.8 µm for each primer, and 0.2 µm of each probe. H5 cDNA positive controls were provided by the CDC and used to quantify each rRT-PCR assay. An ABI 7900 real-time thermocycler was used for all rRT-PCR reactions. The thermocycling parameters for all targets consisted of 50$^0$C for 30 minutes, 95$^0$C for 2 minutes, and 45 cycles with 95$^0$C for 15 seconds, 55$^0$C for 30 seconds.

### Virus culture, isolation, and identification

For virus culture, a 0.2 ml aliquot of each specimen was inoculated onto MDCK cells that had been prepared in sterile 24-well plates, and resulting viruses were reacted with type-specific monoclonal antibodies as previously described [14]. To identify the strains, HAI assay was performed using turkey red blood cells. All isolates were tested against standard reference antisera, which were regularly updated by the CDC.

### Data storage and analysis

Questionnaires consisting of demographic, epidemiology, and clinical data, along with laboratory data (RT-PCR and virus isolation results) and available climate data (rainfall, temperature, and relative humidity provided by the Climatology, Meteorology, and Geophysics Agency, Jakarta, Indonesia) from 18 districts were entered into an Access (Microsoft, Bellevue, WA, USA) database. Data were analyzed using Stata software (Stata Corporation, College Station, TX, USA). Chi-squared test was used for comparison between two proportions of categorical data. Nonparametric tests were used to assess the correlation between numerical and proportional data.



### Human subjects approval

The protocol was approved by the ethical research committees at NIHRD and NAMRU# 2 (DoD protocol 1999-30849).

## RESULTS

During the 5-year surveillance period, a total of 21,030 participants, including outpatients (n=20,012) and inpatients (n=1,018), who presented to surveillance sites with ILI were enrolled. The median age of participants was 17 years (mean age, 21.1 years; range, 1 month to 90 years). Among all participants, 1,851 (8.9%) were aged <2 years, 2,936 (14.1%) were 2-4 years, 4,435 (21.3%) were 5-12 years,

1,220 (5.9%) were 13-17 years, 8,589 (41.3%) were 18-49 years, 1,413 (6.8%) were 50–64 years, and 359 (1.7%) were 65 years and older (Table 1).

Overall, 20.1% (4,236/21,030) of ILI cases throughout the study period tested positive for influenza viruses. The proportion of ILI cases that tested positive for influenza viruses by age-group was highest in school-aged children (Table 1). The percentages of outpatients (20.1%) and inpatients (21.7%) testing positive for influenza viruses were similar. The proportion of seasonal influenza-associated pneumonia, diagnosed by clinicians based on clinical manifestations and chest radiography results, among hospitalized ILI cases sampled was 18% (23/128). Among all ILI cases that tested positive for influenza viruses, influenza A virus and influenza B virus were identified in 64.9% (2,749) and 35.1% (1,487) of cases, respectively. Among the influenza A viruses that were subtyped (n = 2,314 viruses), 64.6% were identified as H3N2, 34.9% as H1N1, and 0.4% as H5N1.

The proportion of ILI cases that tested positive for influenza viruses per year was generally consistent throughout the study period and varied from 18.7% (in 2003) to 23.8% (in 2004). However, the proportion of ILI cases that tested positive for influenza viruses varied considerably across island sites, ranging from Bali (14.2%) and Timor (15.3%) to Papua (25.9%) and Maluku (26.6%). Across the surveillance sites, influenza viruses were detected year round. The distribution of influenza virus types and influenza A virus subtypes varied temporally (Table 2).

**Table 1.** Distribution of influenza specimens testing positive by RT-PCR (by type and influenza A subtype) by age groups. Clinical setting, and location. 2003-2007

| Factor | Total ILI patients tested | Influenza A not further sub typed | | H1N1 | | H3N2 | | H5N1 | | B | | Total | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | % | | % | | % | | % | | % | | % | |
| | | Positive | Positive | Positive | Positive | Positive | Positive | Positive | Positive | Positive | Positive | Positive | Positive |
| Age, years | | | | | | | | | | | | | |
| 0-1 | 1851 | 50 | 2.7 | 24 | 1.3 | 47 | 2.5 | 0 | 0 | 56 | 3 | 177 | 9.6 |
| 2-4 | 2936 | 64 | 2.2 | 122 | 4.2 | 154 | 5.2 | 0 | 0 | 166 | 5.7 | 506 | 17.2 |
| 5-12 | 4435 | 110 | 2.5 | 228 | 5.1 | 278 | 6.3 | 2 | 0.05 | 499 | 11.3 | 1117 | 25.2 |
| 13-17 | 1220 | 32 | 2.6 | 52 | 4.3 | 102 | 8.4 | 2 | 0.2 | 125 | 10.2 | 313 | 25.7 |
| 18-49 | 8589 | 148 | 1.7 | 334 | 3.9 | 768 | 8.9 | 6 | 0.1 | 561 | 6.5 | 1817 | 21.2 |
| 50-64 | 1413 | 20 | 1.4 | 35 | 2.5 | 101 | 7.1 | 0 | 0 | 53 | 3.8 | 209 | 14.8 |
| ≥65 | 359 | 4 | 1.1 | 4 | 1.1 | 20 | 5.6 | 0 | 0 | 16 | 4.5 | 44 | 12.3 |
| Clinical setting | | | | | | | | | | | | | |
| Inpatient | 1018 | 3 | 0.3 | 49 | 4.8 | 86 | 8.4 | 9 | 0.9 | 74 | 7.3 | 221 | 21.7 |
| outpatient | 20012 | 432 | 2.2 | 759 | 3.8 | 1410 | 7.0 | 1 | 0.004 | 1413 | 7.1 | 4015 | 20.1 |
| Sumatra | 2456 | 59 | 2.4 | 132 | 5.4 | 167 | 6.8 | 1 | 0.04 | 188 | 7.6 | 547 | 22.3 |
| Jawa | 10481 | 253 | 2.4 | 388 | 3.7 | 747 | 7.1 | 7 | 0.1 | 639 | 6.1 | 2034 | 19.4 |
| Bali | 1202 | 0 | 0 | 23 | 1.9 | 51 | 4.2 | 1 | 0.1 | 96 | 8.0 | 171 | 14.2 |
| Lombok | 849 | 1 | 0.1 | 28 | 3.3 | 101 | 11.9 | 0 | 0 | 71 | 8.4 | 201 | 23.7 |
| Kalimantan | 1392 | 40 | 2.9 | 43 | 3.1 | 81 | 5.8 | 0 | 0 | 130 | 9.3 | 294 | 21.1 |
| Sulawesi | 1768 | 77 | 4.4 | 43 | 2.4 | 89 | 5.0 | 1 | 0.1 | 116 | 6.6 | 326 | 18.4 |
| Timor | 816 | 1 | 0.1 | 36 | 4.4 | 52 | 6.4 | 0 | 0 | 36 | 4.4 | 125 | 15.3 |
| Maluku | 443 | 0 | 0 | 19 | 4.3 | 56 | 12.6 | 0 | 0 | 43 | 9.7 | 118 | 26.6 |
| Papua | 1623 | 4 | 0.2 | 96 | 5.9 | 152 | 9.4 | 0 | 0 | 168 | 10.4 | 420 | 25.9 |
| Total | 21030 | 435 | 2.1 | 808 | 3.8 | 1496 | 7.1 | 10 | 0.05 | 1487 | 7.1 | 4236 | 20.1 |

**Table 2.** Influenza virus types and influenza A subtypes detected by year

| Year | ILI cases | | RT-PCR | | | |
|---|---|---|---|---|---|---|
| | | | # influenza positive | % of all influenza positive | #isolates | # and (%) of influenza positives with isolates |
| 2003 | 593 | Influenza A | | | | |
| | | H1N1 | 10 | 9.0 | 7 | 7./10 (70) |
| | | H3N2 | 75 | 67.6 | 34 | 34/75 (45.3) |
| | | Influenza B | 26 | 23.4 | 9 | 9/26 (34.6) |
| | | Total | 111/593 | 18.7 | 50 | 50/111 (45) |
| 2004 | 1403 | Influenza A | | | | |
| | | H1N1 | 12 | 3.6 | 8 | 8/12 (66.7) |
| | | H3N2 | 219 | 65.6 | 94 | 94/219 (42.9) |
| | | Influenza B | 103 | 30.8 | 70 | 70/103 (67.9) |
| | | Total | 334/1403 | 23.8 | 172 | 172/334 (51.5) |
| 2005 | 3964 | Influenza A | | | | |
| | | H1N1 | 59 | 7.9 | 22 | 22/59 (37.3) |
| | | H3N2 | 303 | 40.5 | 47 | 47/303 (15.5) |
| | | H5N1 | 1 | 0.1 | | |
| | | Influenza B | 386 | 51.5 | 233 | 233/386 (60.4) |
| | | Total | 749/3964 | 18.9 | 302 | 302/748 (40.4)* |
| 2006 | 6926 | Influenza A | | | | |
| | | A not further tested | 108 | 8.6 | | |
| | | H1N1 | 422 | 33.5 | 196 | 196/422 (46.4) |
| | | H3N2 | 343 | 27.2 | 54 | 54/343 (15.7) |
| | | H5N1 | 3 | 0.2 | | |
| | | Influenza B | 384 | 30.5 | 167 | 167/326 (51.2)** |
| | | Total | 1260/6926 | 18.2 | 417 | 417/1091 (38.2)* |
| 2007 | 8144 | Influenza A | | | | |
| | | A not further tested | 327 | 18.4 | | |
| | | H1N1 | 305 | 17.1 | 89 | 89/305 (29.2) |
| | | H3N2 | 556 | 31.2 | 51 | 51/556 (9.2) |
| | | H5N1 | 6 | 0.3 | | |
| | | Influenza B | 588 | 32.9 | 183 | 183/470 (38.9)** |
| | | Total | 1782/8144 | 21.9 | 323 | 323/1331 (24.3)* |

*Excludes H5N1 viruses and specimens not further tested.

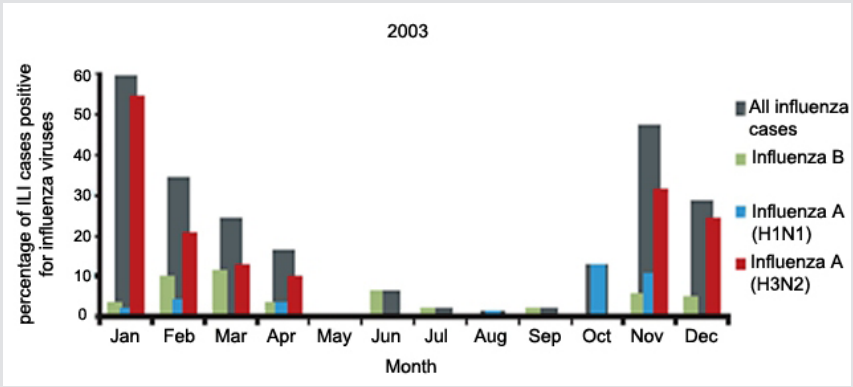
**Denominator differs from all influenza B virus positive specimens detected by RT-PCR

All influenza viral isolates (n=1,264) were first characterized at NAMRU#2 and then a subset (n=473 isolates from 2003 to mid-2006) was further characterized at CDC. In 2003, surveillance was conducted in five sites, and 111 influenza virus infections were identified; influenza A (H3N2) viruses predominated, followed by influenza B and A (H1N1) viruses. The most frequent strains identified were A/Fujian/411/2002-like H3N2, B/Hongkong/330/2001-like (B/Victoria/2/87 lineage, 2002–2003 Northern and 2003 Southern Hemisphere vaccine strain), and A/New Caledonia/20/99-like H1N1 (2002–2003 Northern and 2003 Southern Hemisphere vaccine strain) viruses. The number of surveillance sites expanded during 2004–2007. In 2004, A (H3N2) viruses also predominated, with A/California/7/2004-like H3N2, B/Sichuan/379/99-like, and A/New Caledonia/20/99-like H1N1 (2003-2004 Northern and 2004 Southern Hemisphere vaccine strain) viruses the most frequently detected strains. In 2005, influenza B viruses of the B/Victoria/2/87 line age predominated and most viruses were related antigenically to the reference strain B/Ohio/05/2005. The frequency of circulating A (H3N2) viruses was slightly less than influenza B viruses and most were identified as A/California/7/2004-like. Influenza A (H1N1) viruses were detected at low frequency and all isolates were A/New Caledonia/20/99-like (2004–2005 Northern and 2005 Southern Hemisphere vaccine strain). In 2006, influenza A (H1N1), A (H3N2), and type B viruses were detected at similar frequencies; most H1N1 viruses were related antigenically to A/New Caledonia/20/99-like (2005–2006 Northern and 2006 Southern Hemisphere vaccine strain); the majority of H3N2 viruses were related antigenically to the A/California/7/2004 (2005–2006 Northern and 2006 Southern Hemisphere vaccine strain); and the majority of influenza B viruses belonged to the B/Victoria/2/87-lineage and were identified as B/Malaysia/2506/2004-like. In 2007, influenza A (H3N2) and B viruses were identified in similar frequencies. The majority of H3N2 viruses were related antigenically to A/Wisconsin/67/2005 virus (2006–2007 Northern and 2007 Southern Hemisphere vaccine strain); and the majority of B viruses were of the B/Victoria-lineage viruses and related antigenically to B/Malaysia/2506/2004 virus (2006–2007 Northern and 2007 Southern Hemisphere vaccine strain). Overall, during 2003–2007, influenza A (H3N2) viruses were isolated less frequently (18.7%) than A (H1N1) (39.9%) or B (50.5%) viruses.

Influenza A (H5N1) virus infection was identified in one outpatient and nine inpatients from six surveillance sites (five cases from Tangerang, and one each from Bali, Padang, Jakarta, Makassar, and Yogyakarta). Initial diagnoses in these cases included severe bronchopneumonia, dengue, or typhoid fever. All nine confirmed H5N1 hospitalized cases presented with severe illness late in the clinical course, received late oseltamivir treatment, and all died. One H5N1 case presented with ILI to a hospital outpatient surveillance site, was not suspected with H5N1 at clinical presentation, was neither treated nor hospitalized, and subsequently died. H5N1 virus infection was retrospectively confirmed from a respiratory specimen collected through ILI surveillance. These 10 cases represented 8.5% of all confirmed H5N1 cases (n = 117) identified in Indonesia between 2005 and 2007.

Influenza virus detection by RT-PCR and subsequent viral isolation were higher in ILI patients presenting with a measured temperature of ≥37.8$^0$C (22.6% and 44.7%, respectively) compared to those with a history of feverishness, but without a documented fever at presentation (17.4% and 38.3%). Among 4236 patients who tested positive by RT-PCR, 62.5% were positive in both nasal and throat swab specimens, compared to 19.6% of nasal swabs only, and 16.9% of throat swabs alone. Nasal swabs yielded slightly higher isolation of influenza viruses compared to throat swabs [1225/3043 (40.3%) versus 1155/3054(37.8%), P = 0.04, chi-squared test].

Figure 2 shows the monthly proportion of influenza positive ILI cases identified by RT-PCR. Seasonal peaks in influenza A virus activity, especially with H3N2 virus strains, were observed during December and January, followed by increases in influenza B virus activity during March to May. The seasonality of influenza A virus was largely consistent across all nine islands although some had increased activity during May and July (Sumatra, Maluku, and Papua). Two eastern provinces of Indonesia appeared to have a bimodal peak in influenza activity during December-January and June-July although data were limited (data not shown). We observed a high correlation between the mean proportion of influenza A positives among ILI cases and mean precipitation from 18 districts (Figure 3; r = 0.87). When assessed per district, influenza A virus activity appeared


2003
percentage of ILI cases positive for influenza viruses
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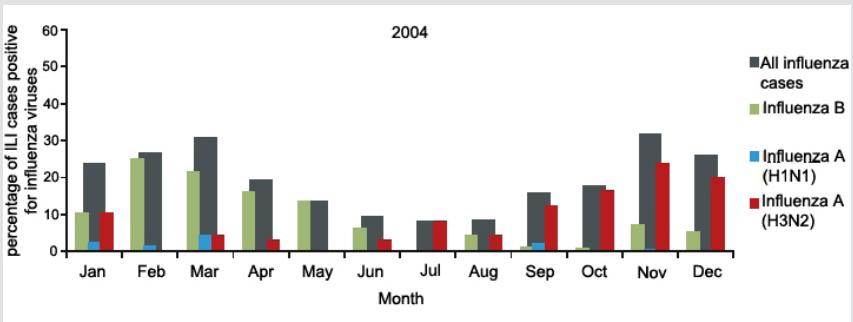
Influenza A (H3N2)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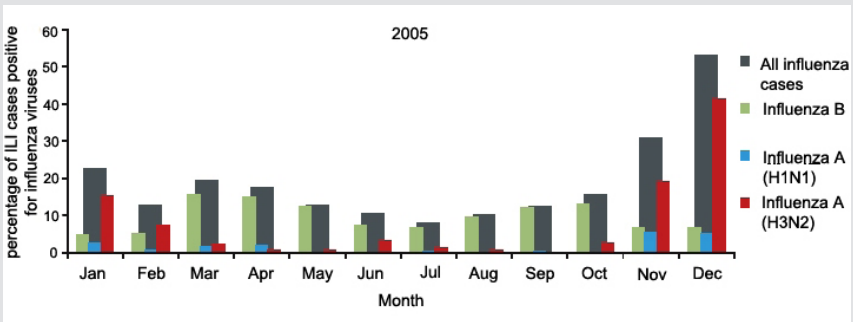
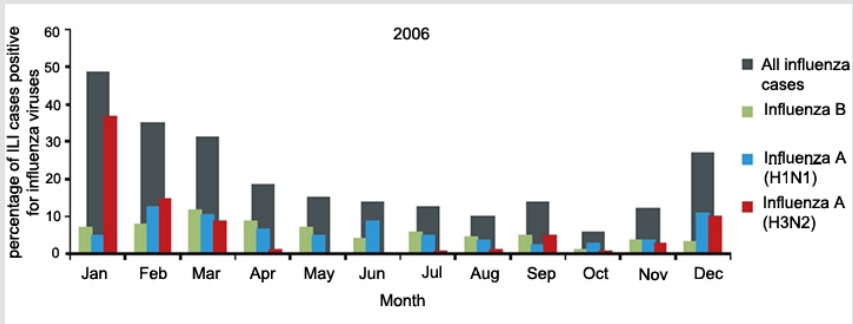
Influenza A (H1N1)
Influenza A (H3N2)


2005
percentage of ILI cases positive for influenza viruses
60
50
40
30
20
10
0
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Month
All influenza cases
Influenza B
Influenza A (H1N1)
Influenza A (H3N2)



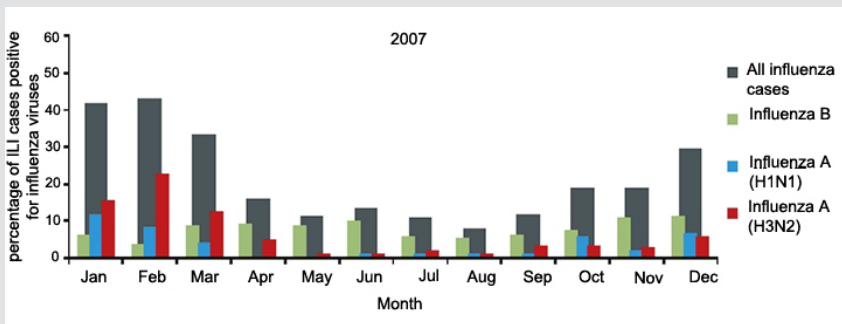




**Figure 2.** Percentage of ILI cases testing positive for influenza viruses by RT-PCR by month, 2003 – 2007

to correlate well with rainfall in 10 districts (in one district, $r > 0.8$; in nine districts, $r = 0.6–0.8$); poor-to-moderate correlation was observed in other districts (in one district, $r = 0.4–0.6$; in two districts, $r = 0.2–0.4$; in three districts, $r = 0–0.2$; and in two districts, $r = 0.01$ to 0.2.) The last five districts are located in Kalimantan and two eastern islands of Indonesia (Maluku and Papua). Influenza A virus activity did not appear to correlate with mean monthly temperature or relative humidity (data not shown). Influenza B virus activity did not appear to be correlated with mean monthly temperature, mean monthly rainfall, or monthly relative humidity for any sites (data not shown).

Retrospective analysis identified a small number of influenza A viruses that were isolated from respiratory specimens collected through surveillance in Indonesia

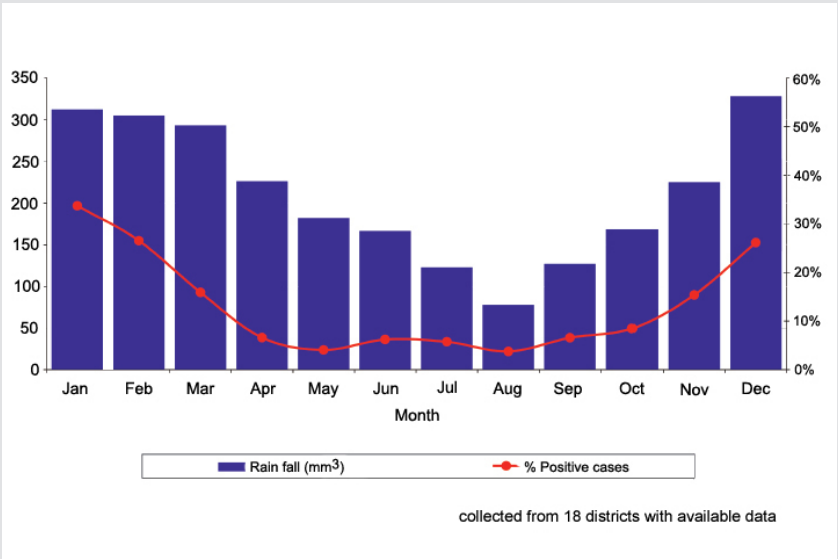



**Figure 3.** Comparison of rainfall precipitation and the proportion of ILI cases that tested positive for influenza A, 2003-2007 indonesia*

before the collection of specimens elsewhere that yielded influenza A (H1N1) and (H3N2) viruses that were subsequently recommended as WHO influenza vaccine strains (Table 3). A/Solomon Islands/3/2006 (H1N1) virus was first identified in August 2006; this antigenic variant virus spread widely and was selected as the Northern Hemisphere H1N1 vaccine component in February 2007 to replace A/New Caledonia/20/99 (H1N1). An H1N1 virus detected in Indonesia during March 2006 was identified as A/Solomon Islands/3/2006-like (in early 2007), suggesting detection of this virus in Indonesia approximately 5 months earlier than the designated WHO Northern Hemisphere vaccine strain. Similarly, a few H3N2 viruses were identified in Indonesia earlier or at the same time as antigenically equivalent H3N2 vaccine strains that were recommended as WHO H3N2 vaccine strains (A/California/7/2004-like and A/Wisconsin/67/2005-like viruses).

**Table 3.** Detection of influenza A virus strains in Indonesia and temporal relations to Global Detection of WHO Designated influenza A vaccine virus strains, 2003-2007

| Influenza vaccine strain | Month, year selected as WHO vaccine strain | Date of collection of influenza vaccine virus | Date of collection of Indonesia virus | Indonesia virus name | Antigenic characterization* |
|---|---|---|---|---|---|
| A/California/07/2004 (H3N2) | February 2005 | Sept 16, 2004 | Sept 16, 2004 | Indonesia/1857/2004 | A/California/07/2004 (H3N2)-like |
| A/Wisconsin/67/2005 (H3N2)-like | February 2006 | Aug 31, 2005 | Feb 18, 2005 | Indonesia/1711/2005 | A/Wisconsin/67/2005 (H3N2)-like |
| A/Solomon islands/03/2006 (H1N1)-like | February 2007 | Aug 21, 2006 | March 3, 2006 | Indonesia/3208/2006 | A/Solomon islands/03/2006 (H1N1)-like |

*With sequence confirmation

## DISCUSSION

Surveillance conducted at sites across the Indonesian archipelago during 2003–2007 identified a substantial proportion of influenza virus infections among patients presenting with influenza-like illness. While influenza activity was detected year-round, the proportion of ILI patients with influenza and predominant viruses varied from year-to-year and geographically. There was evidence for a mixed seasonal distribution of influenza viruses in some parts of eastern Indonesia (Maluku and Papua). However, the observed seasonality of influenza A virus activity among ILI cases at our surveillance sites in most regions of Indonesia, particularly in the western and middle islands which are more densely populated, indicated a peak in December and January, which correlates with the rainy season. Seasonality for influenza B virus was less apparent.

Similar to our surveillance findings, influenza A (H1N1) virus activity was low during 2003–2005 in Australia [15-17], Malaysia [18], and Thailand [19]. However, in 2006, A (H1N1) viruses were the predominant subtype in Malaysia [18], Thailand [19], and Indonesia. In 2007, A (H1N1) activity increased in Australia [20]. Between 2003 and 2007, A (H3N2) virus was the most predominant subtype in

Indonesia and in neighboring countries [15-21]. Similar to our findings, influenza B viruses were less frequently detected than influenza A viruses, but still were identified in a moderately high proportion of ILI cases in Malaysia and Thailand (approximately 20% to 40% each year) [18,19], except in 2005 when B viruses predominated in Malaysia and Indonesia (approximately 52%) [18]. In Australia, influenza B virus activity was variable during 2003–2007 (6% in 2003 to 29% in 2006 and 9% in 2007) [15-17,20,21].

Influenza A and B viruses, including antigenically drifted strains as observed in other countries, were also detected in Indonesia during the surveillance period, and in a few instances were detected earlier than designated WHO representative reference or influenza vaccine strains. A sink-source model in which evolution of influenza A (H3N2) virus strains circulating in the tropics seed winter epidemics in temperate region has been proposed; this model emphasizes the importance of South-East Asia for the emergence of new variants and novel strains [4,5]. Although we did not conduct comprehensive antigenic characterization of influenza viruses circulating in Indonesia or compare our limited results with global data, we believe that improved influenza surveillance in Indonesia, the 4th most populated country, can inform better understanding of the evolution of influenza viruses in South-East Asia.

Given the influenza virus seasonality observed among ILI cases at most Indonesian sentinel sites, use of influenza vaccine in Indonesia should target the December-January winter seasonal peak. For this reason and the similarity with most circulating A (H1N1) and A (H3N2) virus strains during 2003–2007, the timing of the availability of Northern Hemisphere influenza vaccine is best suited for Indonesia. This timing of peak influenza activity is different than that found in other countries in the region which also experience year-round activity, but generally experience a mid-year peak, although the timing of peak activity may vary from year-to-year [18,22-24].

Historically, influenza vaccine use is quite low in Indonesia with fewer than 300,000 doses administered every year [25]. With very limited data on influenza disease burden in Indonesia and no data on cost-effectiveness of influenza vaccination, the Ministry of Health recommends influenza vaccine for high-risk

populations such as the elderly, those with underlying chronic diseases, Hajj pilgrims, and healthcare workers [26]. The main reasons for low influenza vaccine coverage are the cost of vaccine and the wide belief that influenza virus infection only causes mild illness [25]. Oseltamivir is the only antiviral for influenza used in Indonesia, but is not widely available. Although oseltamivir has been provided in limited quantities at government primary health centers and hospitals since 2006, it is not available at pharmacies and its use is restricted to treatment of persons with suspected or confirmed H5N1 virus infection [27].

Among samples tested, the percentage of ILI cases testing positive for influenza viruses was highest among school-age children in Indonesia. While health utilization data for Indonesians of all ages with influenza-like illness are needed, the high burden of influenza among school-age children presenting to surveillance sites is consistent with reports from other countries in the region and underscores the potential of this group as a source of transmission and the importance of influenza vaccination [23,24]. Furthermore, seasonal influenza represented 21.7% of all ILI cases tested in the inpatient setting, among which 18% were associated with pneumonia. Since influenza diagnostic testing is generally not performed as part of clinical management of outpatients or hospitalized patients, the findings in this work can inform physicians in Indonesia who are not aware of the public health impact of seasonal influenza. Furthermore, given the relatively high frequency of influenza among hospitalized ILI patients, both healthcare personnel and other hospitalized patients may be at risk of and contribute to nosocomial influenza virus transmission; the role of influenza vaccination and importance of infection control must be emphasized.

We identified 10 H5N1 cases from patients' respiratory specimens collected at hospital surveillance sites, 8.6% of the 116 H5N1 cases reported in Indonesia during the surveillance period [28]. Nine cases were in suspected H5N1 patients with severe illness admitted to hospitals that were surveillance sites, while one H5N1 case presented with ILI and was not suspected with H5N1. This highlights the value of laboratory-based influenza surveillance to detect human infections with seasonal influenza viruses as well as H5N1 virus [29]. As our surveillance network relied upon a convenience sample of ILI cases among outpatients and inpatients, it is possible that other H5N1 virus infections were missed.

We recognize several limitations to our surveillance findings. First, denominator data on the catchment population for sentinel sites were not collected and data on the total number of outpatient and inpatient visits and total ILI cases were not collected to calculate the incidence of ILI and influenza. We utilized a convenience sample of ILI cases, not a systematic sample. Therefore, influenza disease burden could not be estimated. Nevertheless, we documented a substantial proportion of outpatients and hospitalized patients with influenza. Second, we did not follow the outcomes of our inpatients to estimate seasonal influenza-associated mortality. Third, sentinel sites were selected based on the population density. More sentinel sites existed on two islands, Sumatra and Java in the West, and our findings may not be representative of all of Indonesia, including less populous eastern islands. Finally, the yield of viral isolation was lower over the surveillance period because of cold-chain issues and difficulties in isolating H3N2 viruses. However, as we utilized RT-PCR as the primary method of identification, we believe that our findings accurately reflect influenza virus activity among our surveillance sites.

In conclusion, our findings demonstrate that influenza is an important public health problem among outpatients and hospitalized ILI patients in Indonesia. This has implications for understanding the public health impact of influenza in tropical climates as well as the development of policies for the use of influenza vaccines in Indonesia. In addition to strengthening and improving epidemiological data collection for patients with influenza, collecting more data on influenza disease burden is needed, especially among hospitalized patients and high-risk groups for influenza complications. Also, data are needed on the economic impact of influenza and the role of climactic factors (such as absolute humidity, rainfall, and temperature) in influenza activity and seasonality in Indonesia.



## ACKNOWLEDGEMENTS

We thank all of the volunteers, clinicians, and nurses from participating sentinel surveillance sites and the administrative and laboratory staff at NIHRD, and NAMRU#2, especially Chairin Ma'roef, and Ungke Antonjaya. We thank the

Influenza Division, CDC for providing updated influenza primers, probes, and antisera, laboratory support for the field and laboratory surveillance activities. We also thank Jens Levy (CDC Thailand) for critical review of the manuscript. We dedicate this work to the memory of our beloved colleague Dr. Endang R. Sedyaningsih.

## DISCLAIMER

The author(s) declare that they have no conflict of interest. This work was funded in part by grants from the U.S. Department of Defense Armed Forces Health Surveillance Center, Division of Global Emerging Infections Surveillance (AFHSC / GEIS) and the U.S. Centers for Disease Control and Prevention (CDC). The views expressed are those of the authors and do not necessarily represent the official policies of the CDC, the U.S. Department of Defense or the Department of the Navy.

## References


1. Monto AS (2008) Epidemiology of influenza. Vaccine: D45-D48.
2. Viboud C, Alonso WJ, Simonsen L (2006) Influenza in tropical regions. PLoS Med 3: e89.
3. Leo YS, Lye DC, Chow A (2009) Influenza in the tropics. Lancet Infect Dis 9: 457-458.
4. Rambaut A, Pybus OG, Nelson MI, Viboud C, Taubenberger JK, et al. (2008) The genomic and epidemiological dynamics of human influenza A virus. Nature 453: 615-619.
5. Russell CA, Jones TC, Barr IG, Cox NJ, Garten RJ, et al. (2008) The global circulation of seasonal influenza A (H3N2) viruses. Science 320: 340-346.
6. Sedyaningsih ER, Isfandari S, Setiawaty V, Rifati L, Harun S, et al. (2007) Epidemiology of cases of H5N1 virus infection in Indonesia, July 2005-June 2006. J Infect Dis 196: 522-527.
7. Kandun IN, Tresnaningsih E, Purba WH, Lee V, Samaan G, et al. (2008) Factors associated with case fatality of human H5N1 virus infections in Indonesia: a case series. Lancet 372: 744-749.
8. Simmerman JM, Lertiendumrong J, Dowell SF, Uyeki T, Olsen SJ, et al. (2006) The cost of influenza in Thailand. Vaccine 24: 4417-4426.
9. Simmerman JM, Uyeki TM (2008) The burden of influenza in East and South-East Asia: a review of the English language literature. Influenza Other Respir Viruses 2: 81-92.
10. Beckett CG, Kosasih H, Ma'roef C, Listiyaningsih E, Elyazar IR, et al. (2004) Influenza surveillance in Indonesia: 1999-2003. Clin Infect Dis 39: 443-449.
11. Zhang WD, Evans DH (1991) Detection and identification of human influenza viruses by the polymerase chain reaction. J Virol Methods 33: 165-189.
12. Hobson D, Curry R, Beare A, A W-G (1972) The Role of serum haemagglutination-inhibition antibody in protection against challenge infection with influenza A2 and B viruses. J Hyg 70: 767–777.
13. World Health Organization (2012) Recommendations and laboratory procedures for detection of avian influenza A(H5N1) virus in specimens from suspected human cases http://www.who.int/influenza/resources/documents/RecAllabtestsAug07.pdf 1 may 2012.
14. Gaush CR, Smith TF (1968) Replication and plaque assay of influenza virus in an established line of canine kidney cells. Appl Microbiol 16: 588-594.
15. Yohannes K, Roche P, Hampson A, Miller M, Spencer J (2004) Annual report of the National Influenza Surveillance Scheme, 2003. Commun Dis Intell 28: 160-168.
16. Li J, Hampson A, Roche PW, Yohannes K, Spencer JD (2005) Annual report of the

National Influenza Surveillance Scheme, 2004. Commun Dis Intell 29: 125-136.
17. Firestone SM, Barr IG, Roche PW, Walker JC (2006) Annual report of the National Influenza Surveillance Scheme, 2005. Commun Dis Intell 30: 189-200.
18. Saat Z, Abdul Rashid TR, Yusof MA, Kassim FM, Thayan R, et al. (2010) Seasonal influenza virus strains circulating in Malaysia from 2005 to 2009. Southeast Asian J Trop Med Public Health 41: 1368-1373.
19. Chittaganpitch M, Supawat K, Olsen SJ, Waicharoen S, Patthamadilok S, et al. (2012) Influenza viruses in Thailand: 7 years of sentinel surveillance data, 2004-2010. Influenza Other Respir Viruses 6: 276-283.
20. Owen R, Barr IG, Pengilley A, Liu C, Paterson B, et al. (2008) Annual report of the National Influenza Surveillance Scheme, 2007. Commun Dis Intell 32: 208-226.
21. O'Brien K, Barr IG (2007) Annual report of the National Influenza Surveillance Scheme, 2006. Commun Dis Intell 31: 167-179.
22. Simmerman JM, Chittaganpitch M, Levy J, Chantra S, Maloney S, et al. (2009) Incidence, seasonality and mortality associated with influenza pneumonia in Thailand: 2005-2008. PLoS One 4: e7776.
23. Blair PJ, Wierzba TF, Touch S, Vonthanak S, Xu X, et al. (2010) Influenza epidemiology and characterization of influenza viruses in patients seeking treatment for acute fever in Cambodia. Epidemiol Infect 138: 199-209.
24. Nguyen HT, Dharan NJ, Le MT, Nguyen NB, Nguyen CT, et al. (2009) National influenza surveillance in Vietnam, 2006-2007. Vaccine 28: 398-402.
25. Tan. (2011) Dokter Keluarga bisa perluas cakupan imunisasi dewasa http://www.majalah-farmacia.com/rubrik/onenews.asp?IDNews=2194 1 may 2012. Farmacia 10: 64.
26. Dirjen BUK (2003) Imunisasi influenza untuk anak dan dewasa. http://buk.depkes.go.id/index.php?option=com_docman&task=doc_details&gid=258&Itemid=58 HTA Indonesia 19: 1-22.
27. Narulita Y (2008) Gambaran pelaksanaan majaemen logistik antiviral dalam penanganan flu burung di propinsi Banten tahun 2005–2008. Undergraduate thesis, for Public Health Faculty; University of Indonesia: 1-86.
28. World Health Organization (2007) Avian influenza – situation in Indonesia – update 28 26 December 2007 http://www.who.int/csr/don/2007_12_26/en/index.html 1 may 2012.
29. Brooks WA, Alamgir AS, Sultana R (2009) Avian influenza virus A (H5N1), detected through routine surveillance, in child, Bangladesh. Emerg Infect Dis 15: 1311–1313.

# Chapter 9

## Evidence of human hantavirus infection and zoonotic investigation of hantavirus prevalence in rodents in western Java, Indonesia

*Vector-borne and zoonotic Diseases, 2011*

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## ABSTRACT

During febrile surveillance in the western Java City of Bandung, Indonesia, a patient with clinical symptoms consistent with hantavirus infection was found to have elevated titers of hantavirus-specific immunoglobulin M (IgM) and IgG antibodies. A subsequent epizoological investigation demonstrated a higher prevalence of hantavirus IgG antibodies in rodents trapped in the vicinity of the patient's home compared with rodents from a control area (13.2% vs. 4.7%, p=0.036). The Old World Seoul hantavirus was detected by reverse transcriptase – polymerase chain reaction in the organs of 71% of the seropositive rodents tested. This is the first report of a Seoul virus infection in Indonesia supported by clinical, serological, and epizoological evidences. These findings suggest that hantavirus infection should be on the clinical differential diagnosis when acutely ill febrile patients report for care in western Java.


## INTRODUCTION

Hantaviruses are negative-stranded RNA viruses that belong to the *Bunyaviridae* family. The Old World hantaviruses include the Seoul, Dobrava, and Puumala viruses [1]. They are rodent-borne viruses and the causative agents of two disease syndromes, hemorrhagic fever with renal syndrome (HFRS), more prominent following infection with Old World hantaviruses, and hantavirus pulmonary syndrome (HPS), which is associated with New World hantaviruses. Hantaviruses cause chronic inapparent infections in rodents throughout the Americas, Asia, and Europe. Each hantavirus has a specific reservoir host with a limited geographic distribution. The common reservoirs for Hantaan (HTN), Seoul, Dobrava, Puumala, and Sin Nombre viruses are *Apodemus agrarius*, *Rattus norvegicus*, *Apodemus flavicollis, Clethrionomys glareolus*, and *Peromyscus maniculatus*, respectively [1]. Humans can become infected with hantaviruses through contact with urine, saliva, or feces from infected rodents, and evidence of HTN and Seoul virus infections have been reported in Southeast Asia. In Indonesia, the presence of hantavirus [2] and Seoul virus [3] antibodies in rodents has been demonstrated. However, reports of hantavirus infections in humans are limited. In 2001, acute sera from 2 of 25 suspected dengue patients

from Yogyakarta, central Java, were positive for hantavirus immunoglobulin M (IgM) and IgG as determined by immunofluorescence assay [4]. In separate studies, hantavirus IgM antibodies were found in 10 of 94 [5] or 20 of 118 [6] suspected dengue patients from Semarang and Yogyakarta, central Java. In this report, we present clinical findings from a patient in western Java with elevated titers of IgM and IgG antibodies against hantavirus and demonstrate evidence of Seoul virus infection in rodents captured near the patient's home. This is the first report of a Seoul virus infection in Indonesia supported by clinical, serological, and epizoological evidences.

## MATERIALS AND METHODS

### PATIENT ENROLLMENT

Hospital-based surveillance for hantavirus was conducted at two large hospitals in Bandung, West Java, Indonesia, from September 2004 to August 2005. Patients eligible for enrollment were hospitalized patients >10 years old who met the clinical criteria of suspected HFRS or HPS (acute renal failure with unknown etiology or fever with at least one of the following symptoms: hemorrhagic manifestations, platelet count <100,000/$mm^3$, renal insufficiency, liver dysfunction of unknown etiology, and/or noncardiogenic pulmonary edema). After obtaining informed consent, blood samples were taken during admission and when discharged or at least a week later. Samples were first screened by reverse transcriptase–polymerase chain reaction (RT-PCR) and serology for evidence of acute dengue infection. Patients RT-PCR positive for dengue or with dengue IgM results that increased between acute and convalescent samples were not tested further. This study was reviewed and approved by the Ethical Commission of the National Institute of Health Research and Development, Indonesian Ministry of Health, Jakarta, Indonesia, and by the Institutional Review Board for the ethical conduct of research on human subjects at the U.S. Naval Medical Research Unit No. 2, Jakarta, Indonesia.

## RODENT TRAPPING

Rodents were trapped for three consecutive nights at each study site. Domestic area trapping was conducted by placing one Sherman (H.B. Sherman Traps, Inc.) and one Tomahawk (Tomahawk Live Trap Co.) trap inside the house and one Tomahawk trap outside the house. Peridomestic trapping was conducted by placing Tomahawk traps at 5-m intervals in a straight line. All rodents were euthanized, and field identification of the animal species was made by a mammalogist. Blood and tissue samples were collected from the rodents and placed in liquid nitrogen. All aspects of animal use were conducted using protocols approved by the U.S. Naval Medical Research Unit No. 2 Institutional Animal Care and Use Committee and the National Institute of Health Research and Development, Indonesian Ministry of Health.

## SEROLOGY

Human serum samples were tested for the presence of dengue-specific IgM using a commercially available kit (Focus Diagnostics) according to the manufacturer's instructions. Human serum samples were tested for the presence of IgM and IgG antibodies against hantavirus using a commercially available ELISA kit (Focus Diagnostics) according to the manufacturer's instructions and by an in-house ELISA using HTN virus antigen from HTN virus infected Vero E6 cell cultures (kindly provided by Dr. Ross Graham, USAMRID). A capture ELISA was run to detect the presence of IgM from human samples. Briefly, samples diluted 1:100 in serum dilution buffer (phosphate-buffered saline, 0.1% Tween-20, and 5% Bacto skim milk) were added to 96-well microtiter plates (Immulon 2; Dynex Technologies) coated with anti-human IgM antibodies (1:500; Kirkegard and Perry). After incubation and washing, HTN antigen diluted 1:6 in 2% normal human serum was added. After incubation and washing, anti-HTN rabbit IgG (1:2000), horseradish peroxidase-conjugate anti-rabbit IgG (1:2000), and ABTS substrate (Kirkegard and Perry) were used to detect antigen–antibody binding.

An indirect ELISA was used to detect anti-HTN IgG from human and animal samples. Horseradish peroxidase conjugated anti-human IgG (Fc specific; Axell), anti-mouse IgG, or anti-rat IgG (Kirkegard and Perry) were used depending upon the species tested. A panel of normal sera was used to determine the cutoff. A sample was considered positive if its optical density (OD) value exceeded the mean plus 3 standard deviations of the normal sera. The endpoint ELISA titers were determined by retesting ELISA-positive samples at serial fourfold dilutions beginning at 1:100. The largest dilution giving a positive result was considered the endpoint titer.

## MOLECULAR ANALYSIS

RNA was extracted from human serum samples using the QIAamp Viral RNA kit (Qiagen) or High Pure RNA Tissue kit (Roche Applied Science) for tissue samples, according to the manufacturer's protocol.

RT-PCRs to detect dengue virus were performed as previously described [7]. RT-PCRs to detect hantaviruses were performed using primers specific for each genus, HTN, Seoul, Dobrava, Puumala, and Sin Nombre, as previously described [8].

A hantavirus real-time quantitative RT-PCR protocol was also performed in separate reactions using hantavirus group-specific primers and probes specific for Dobrava, HTN, Puumala, and Seoul viruses as previously described [9]. Positive results were confirmed by sequencing. Contigs were aligned using Sequencher 4.9, and BLAST was performed against the GenBank database.


**Figure 1.** Map of study location

## RESULTS

Hospital-based surveillance for hantavirus was conducted at two large hospitals in Bandung, West Java, Indonesia (Figure 1), from September 2004 to August 2005. Patients eligible for enrollment were hospitalized patients >10 years old who met the clinical criteria of suspected HFRS or HPS (acute renal failure with unknown etiology or fever with at least one of the following symptoms: hemorrhagic manifestations, platelet count <100,000/mm$^3$, renal insufficiency, liver dysfunction of unknown etiology, and/or noncardiogenic pulmonary edema). A total of 406 hospitalized febrile cases were enrolled in the study. Patients were first screened for evidence of recent dengue infection using RT-PCR and serology. Patients who were negative for dengue infection were then tested for evidence of hantavirus infection using RT-PCR and serology. The

majority of patients (311/76.6%) had evidence of recent dengue infection, and one patient was positive for high titer hantavirus antibodies. Details from the patient with high titer hantavirus antibodies are described below.

A 25-year-old previously healthy man presented for treatment at a hospital in Bandung, West Java, Indonesia, on January 5, 2005. The patient suffered from a sudden onset of fever of 5 days in duration, headache, nausea, vomiting, and abdominal discomfort and complained of a loss of appetite and malaise. He lived in a rural area near Bandung and reported no history of travel outside the immediate area. The patient was feverish ($39^0$C) and had a positive tourniquet test, but no conjunctivitis. He was eupneic, with a normal blood pressure (120/80 mmHg) and pulse rate ($80/min^{-1}$). Routine laboratory blood screening showed a normal leukocyte count (9600mm_3), hematocrit (50%), blood urea nitrogen (25mg/dL), and creatinine (0.9 mg/dL). The patient's platelet count ($42{,}000mm^{-3}$), blood protein (5.8 g/dL), and albumin levels (3.3 g/dL) were low and his serum transaminases elevated (SGOT:130 U/L and SGPT: 76U/L). The following day (day 6 of illness) his temperature decreased ($38^0$C), and his platelet count dropped slightly ($38{,}000mm^{-3}$) while his hematocrit remained stable (51%). On day 7 of illness, the patient had defervescence ($38^0$C), and his platelet count increased to $80{,}000mm^{-3}$. On day 8 of illness, his platelet count was above $100{,}000mm^{-3}$, and the patient was discharged from the hospital. Blood chemistry tests repeated on day 17 revealed normal blood urea nitrogen (24mg/dL), normal creatinine (0.9 mg/dL), decreasing serum transaminases (SGOT: 44U/L and SGPT: 46U/L), and normal total protein (6.3 g/dL) and albumin (3.7 g/dL).

Sera were collected on illness days 8 and 17 for diagnostic tests. A commercially available ELISA kit (Focus Technologies) detected high and increasing levels of anti-hantavirus IgM (index >1.1=positive; day 8 sample index=18.5 and day 17 sample index=20.6) and high levels of anti-hantavirus IgG (index >1.1=positive; day 8 and 17 sample index=23.2). An in-house ELISA also detected high and increasing titers of anti-hantavirus IgM (day 8, 1:1600; day 17, 1:6400) and IgG (day 8, 1:1600; day 17, 1:6400). A diagnosis of hantavirus infection was reported to hospital officials.

To follow-up on the suspected case and determine the prevalence of disease among rodents in the area, a field study was conducted at 10 months after the patient presented to the hospital. Rodents were trapped around the patient's home, which was also his working space (case area), and around a nonhantavirus patient's home (control area) 4 km away. A total of 245 rodents were trapped, 159 from the case area and 86 from the control area. *R. norvegicus* was the most predominant in both areas (74.8% and 69.8%), followed by *Rattus tanezumi* (22.6% and 22.1%) and *Mus musculus* (2.5% and 8.1%) (Table 1).

**Table 1.** Distribution of trapped rodents

| Rodent species | Case area | | Control area | |
|---|---|---|---|---|
| | Number of trapped rodents | Number of hantavirus IgG-positive rodents | Number of trapped rodents | Number of hantavirus IgG-positive rodents |
| *Rattus norvegicus* | 119 | 20/119 (16.8%) | 60 | 4 (6.7%) |
| *Rattus tanezumi* | 36 | 1/36 (2.8%) | 19 | 0 |
| *Mus musculus* | 4 | 0 | 7 | 0 |
| Total | 159 | 21/159 (13.2%) | 86 | 4/86 (4.7%) |

IgG, immunoglobulin G.

Sera from trapped rodents in the case area and the control area were tested for anti-hantavirus IgG. A higher percentage of rodents positive for hantavirus-specific IgG was detected from the case area when compared with the control area (13.2% vs. 4.7%; chi-square test, p=0.036). Twenty-four of the positive rodents were *R. norvegicus* (24/179=13.4%), whereas one was *R. tanezumi* (1/55=1.8%) (Table 1). Within the case area, there was a difference in the mean distance from seropositive rats to the patient's home compared with the mean distance from seronegative rats to the patient's home (49.9 vs. 77.5m; chi-square test, p=0.043) (Figure 2).

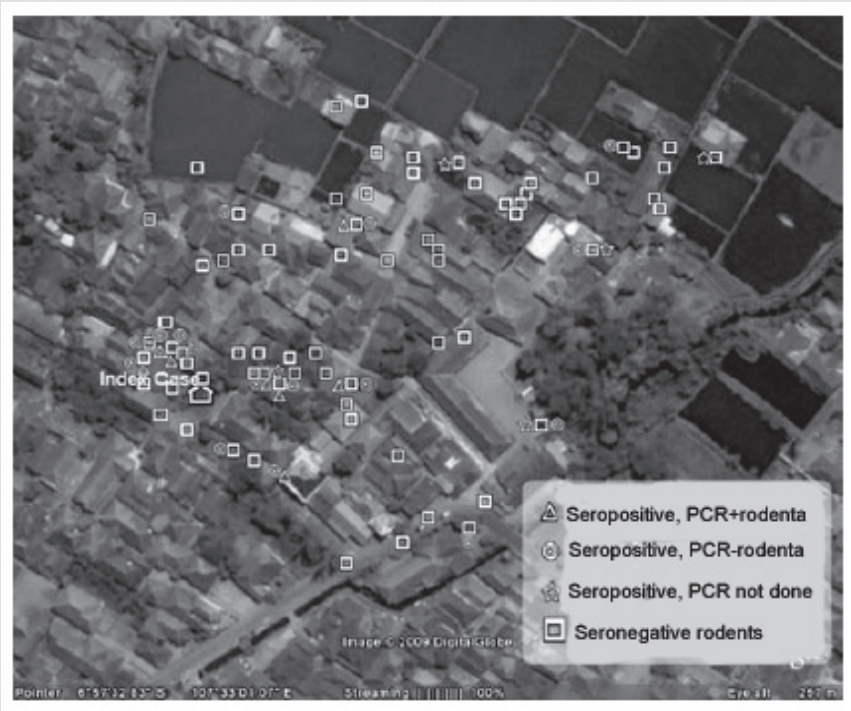


**Figure 2.** Map of patient's home and distribution of trapped rodents

RT-PCR was conducted on lung, kidney, spleen, and liver samples from 13 randomly chosen, IgG-positive rodents from the case area, and the 4 IgG-positive rodents from the control area. Seoul virus was amplified in 9 of the 13 rodents selected from the case area and in all 4 of the rodents selected from the control area. Seoul virus was most commonly detected in the lungs (9/17, 52.9%), followed by the spleen (6/17, 35.3%) and kidney (6/17, 35.3%), and no rodents had RT-PCR–positive livers (Table 2).

**Table 2.** Rat organ positive in reverse transcriptase-polymerase chain reaction for Seoul virus

| | Lungs | Liver | Spleen | Kidney | Any |
|---|---|---|---|---|---|
| Control area | 3/4 | 0/4 | 0/4 | 1/4 | 4/4 |
| Case area | 6/13 | 0/13 | 6/13 | 5/13 | 9/13 |
| Total | 9/17 (52.9%) | 0/17 (0%) | 6/17 (35.2%) | 6/17 (35.2%) | 13/17 (76.5%) |

Sequence analysis of a 225-bp region of the S segment from two specimens collected in the case area demonstrated 99% identity with the S segment of a Seoul virus isolated from an *R. norvegicus* in Singapore (GQ 274945 Seoul virus isolate Singapore/06).

## DISCUSSION

Hantavirus surveillance was conducted in hospitalized patients in West Java, Indonesia, between September 2004 and August 2005. During this time, the majority of enrolled patients had evidence of recent dengue infection, and one patient had serological evidence of hantavirus infection. In this patient, high and increasing titers of antibodies against hantavirus were detected; however, direct determination of the specific hantavirus serotype was not possible as the patient's PCR results were negative and the facilities required to perform the necessary neutralization assays were not available. To further investigate this case, an epizoological investigation was conducted. Results of this investigation demonstrated a higher seroprevalence of hantavirus-specific antibodies in rodents trapped near the patient's home/work compared with rodents trapped in a control area, and Seoul virus was detected in most of the seropositive rodents. Although previous molecular evidence had demonstrated the presence of Seoul virus in rodents collected from central Jakarta [3], this is the first report of its presence in Bandung, West Java. Clinical data also support the diagnosis of Seoul virus infection, as Seoul virus infection is milder than HTN virus infection, and is frequently accompanied by hepatic involvement [1,10] with no or minimal renal disease [11,12].

Clinically mild hantavirus infection is undifferentiated from other viral or bacterial infections, especially dengue, which is endemic in Indonesia. Because many suspected dengue cases are not laboratory confirmed, hantavirus may represent the true etiology of some of these cases. In central Java, hantavirus IgM has been previously reported in up to 17% of suspected dengue cases [4-6]; however, this work in western Java detected evidence of hantavirus infection in <1% of the patients tested. The differences in the reported prevalences between these two locations could be due to differences in methodology or a true difference in prevalence between these two locations. Thus, to determine the prevalence of this disease among febrile patients throughout Indonesia, additional systematic studies should be conducted.

## ACKNOWLEDMENT

This study was supported by the U.S. Army Medical Research Institute for Infectious Diseases.

### DISCLAIMER

The opinions or assertions expressed herein are the private views of the authors and are not to be construed as representing those of the U.S. Navy, the Department of Defense, or the Indonesian Ministry of Health.

### DISCLOSURE STATEMENT

No competing financial interests exist.

## References


1. Hart CA, Bennett M (1999) Hantavirus infections: epidemiology and pathogenesis. Microbes Infect 1: 1229-1237.
2. Ibrahim IN, Sudomo M, Morita C, Uemura S, Muramatsu Y, et al. (1996) Seroepidemiological survey of wild rats for Seoul virus in Indonesia. Jpn J Med Sci Biol 49: 69-74.
3. Plyusnina A, Ibrahim IN, Winoto I, Porter KR, Gotama IB, et al. (2004) Identification of Seoul hantavirus in Rattus norvegicus in Indonesia. Scand J Infect Dis 36: 356-359.
4. Praseno HN (2008) Hantavirus infection in clinically suspected dengue fever patients. Berkala Ilmu Kedokteran 40.
5. Groen J, Suharti C, Koraka P, van Gorp EC, Sutaryo J, et al. (2002) Serological evidence of human hantavirus infections in Indonesia. Infection 30: 326-327.
6. Suharti C, van Gorp EC, Dolmans WM, Groen J, Hadisaputro S, et al. (2009) Hanta virus infection during dengue virus infection outbreak in Indonesia. Acta Med Indones 41: 75-80.
7. Lanciotti RS, Calisher CH, Gubler DJ, Chang G-J, Vorndam AV (1992) Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. Journal of clinical microbiology 30: 545-551.
8. Dekonenko A, Ibrahim MS, Schmaljohn CS (1997) A colorimetric PCR-enzyme immunoassay to identify hantaviruses. Clin Diagn Virol 8: 113-121.
9. Aitichou M, Saleh SS, McElroy AK, Schmaljohn C, Ibrahim MS (2005) Identification of Dobrava, Hantaan, Seoul, and Puumala viruses by one-step real-time RT-PCR. J Virol Methods 124: 21-26.
10. Chan YC, Wong TW, Yap EH, Tan HC, Lee HW, et al. (1987) Haemorrhagic fever with renal syndrome involving the liver. Med J Aust 147: 248-249.
11. Peters CJ, Simpson GL, Levy H (1999) Spectrum of hantavirus infection: hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome. Annu Rev Med 50: 531-545.
12. Chandy S, Boorugu H, Chrispal A, Thomas K, Abraham P, et al. (2009) Hantavirus infection: a case report from India. Indian J Med Microbiol 27: 267-270.

# Chapter 10

## Short report
## West Nile virus documented in Indonesia from acute febrile illness specimens

*AM J Tropical Medicine Hygiene, 2014*

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## ABSTRACT

We report the presence of West Nile virus in a cryopreserved, dengue-negative serum specimen collected from an acute fever case on Java in 2004-2005. The strain belongs to genotype lineage 2, which has recently been implicated in human outbreaks in Europe.

The Indonesian archipelago has been predicted to be a 'hotspot' for the emergence of zoonotic and vector-borne pathogens [1]. Dengue (DENV), chikungunya (CHIKV), and Japanese encephalitis (JEV) are some of the arthropod-borne viruses (arboviruses) known to cause febrile illness in Indonesia but it is suspected that other, relatively uncommon arboviruses are also causing disease. Currently, there is only limited data on the etiology of febrile illnesses in Indonesia [2]. As part of an effort to build capacity to detect the etiologies of underlying acute febrile illnesses in Indonesia, we have begun an analysis of cryopreserved specimens collected in an earlier study of acute febrile illness which had previously tested negative for hantaviruses and DENV.

Archived samples were selected from an acute febrile illness study that enrolled hospitalized suspected hantavirus patients at two hospitals in Bandung, West Java, Indonesia during 2004-2005 [3]. Samples were collected from patients $\geq$ 10 years of age with fever of unknown etiology and at least one of the following symptoms: 1) hemorrhagic manifestations, 2) platelet count < 100,000/mm$^3$, 3) renal insufficiency, 4) liver dysfunction, or 5) non-cardiogenic pulmonary edema. Serum samples were collected from patients at admission to the hospital and at discharge. Collections were made under institutional review board approvals from the National Institute of Health Research and Development, Indonesian Ministry of Health and the U.S. Naval Medical Research Unit No. 2, Jakarta, Indonesia. The samples were originally tested for hantavirus and dengue using reverse transcription-polymerase chain reaction (RT-PCR) and in-house and commercial immunoglobulin M (IgM) and IgG enzyme-linked immunosorbent assay (ELISA) tests (Focus Diagnostics, Cypress, CA).

Of 406 cases enrolled, 249 had evidence of recent DENV infection and one had

evidence of hantavirus infection. Infecting etiologies for the remaining 157 cases were negative for both DENV and hantavirus. We tested 154 acute specimens from these cases, which had been preserved at -80$^0$C, for other arboviruses. Initial testing was performed by using group/family specific primers inconventional RT-PCR assays followed by gel electrophoresis of the resulting amplicons [4]. The flavivirus group primer testing resulted in four positive samples (265-bp amplicon products) which were further tested with virus specific RT-PCR reactions and immunofluorescent assay of Vero cells infected with either DENV or JEV. The purified nucleic acid of one flavivirus positive sample that was negative for both DENV and JEV in the additional testing was subjected to nucleotide sequencing at the Eijkman Institute with the same primers used to generate the PCR product. A 242 basepair sequence from the NS5 gene was generated from the original 265 bp PCR amplicon. Genetic comparisons revealed the closest match (99% nucleotide identity) with the first West Nile virus (WNV) strain isolated (B956), an isolate from Uganda within lineage 2 [5]. Phylogenetic analyses confirmed the relationship of the Indonesian strain with other lineage 2 WNV sequences (Figure 1).

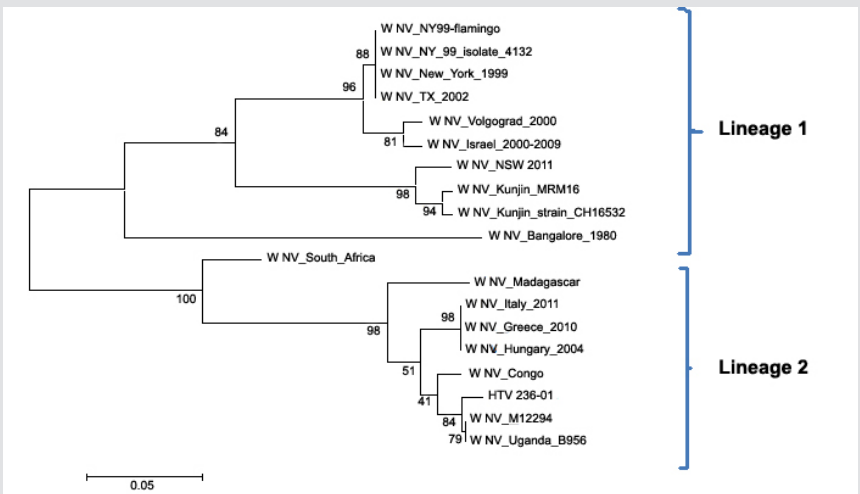


**Figure 1.** A 184 ns5 base-pair nucleotide sequence fragment from Bandung, Indonesia. Sample HTV236-01 was aligned with other WNV sequences from GenBank using BioEdit. The Maximum Likelihood tree was constructed by using software MEGA5. Bootstrap values are expressed as a percentage of 1,000 replicates.

The WNV positive sample came from a 15 year old boy admitted for systemic febrile illness with epistaxis, gastrointestinal symptoms, elevated serum transaminases, leucopenia and thrombocytopenia. No neurological symptoms were reported and the patient was discharged after full recovery. Culture of the serum sample was attempted; however, the sample did not produce cytopathology in Vero cells propagated for10 days.

West Nile Virus, a zoonotic, mosquito-transmitted arbovirus belonging to the *Flaviviridae* family, is reported to be the most common cause of epidemic viral encephalitis in the United States. Phylogenetic analysis has supported the presence of two major genetic lineages. The lineage 1 viruses have typically been associated with large outbreaks and thus are considered to be more virulent than the lineage 2 strains [6]. However, several recent outbreaks in Europe have been caused by lineage 2 WNV strains [7-9]. Thus, it is possible that more recent lineage 2 strains are emerging with increased levels of virulence. The Indonesian strain might follow this trend but data regarding neurovirulence from Indonesia are lacking. The close phylogenetic relationship of the Indonesian strain with those from Uganda rather than strains from Australia is somewhat unexpected as there is relatively less movement of people and goods between Africa and Indonesia. Grouping of isolates, however, does not necessarily correlate with the geographic distribution of the virus [10] and may be a further indicator of the recent widespread movement of pathogenic arboviruses.

The WNV is considered a serious threat to public health and known to cause large outbreaks of epidemic encephalitis in Europe and North America with significant morbidity and mortality [11,12]. WNV has not been previously isolated in Indonesia, but a serological study detected the presence of antibody to this virus in Lombok, Indonesia [13]. Our study associates detection of WNV nucleic acid in Indonesia with human illness. Given that these samples were collected several years ago, the possibility that WNV may be currently circulating in Indonesia warrants further study. The clinical spectrum caused by WNV varies dramatically from inapparent infection (approximately 80% of cases) to non-neurological febrile illness (~ 20% of cases), to the most severe form including neuroinvasive disease (<1%) [14]. Neuroinvasive disease cases have a mortality rate of about 10% and often display chronic manifestations.

There has been limited laboratory capacity in Indonesia to detect arboviruses other than dengue and the role of CHIKV, WNV, JEV and other vector-borne viruses, such as Zika virus, as causes of febrile illness or more serious encephalitis has likely been underestimated. Development of rapid and simple molecular diagnostic tests combined with the establishment of dedicated research facilities in Indonesia will lead to an increased understanding of both endemic and emerging pathogens. With this recent detection of WNV in a febrile human in Indonesia, it is clear that WNV should be considered a serious threat to public health in Southeast Asia. Enhanced surveillance studies in humans, vectors and animals as well as epidemiological surveys are warranted in Indonesia.

## ACKNOWLEDGEMENTS

This study was supported by USAID Emerging Pandemic Threat (EPT) Program and the US Centers for Disease Control and Prevention. We would like to acknowledge Universitas Padjadjaran, Bandung, the National Institute of Health Research and Development, Ministry of Health; and U.S. Naval Medical Research Unit No. 2, Jakarta, Indonesia for their support in conduction of research and specimen archiving. The findings and conclusions in this report are those of the authors only, and do not necessarily reflect the views of their respective institutions.

## References


1. Rosenberg R, Johansson MA, Powers AM, Miller BR (2013) Search strategy has influenced the discovery rate of human viruses. Proc Natl Acad Sci USA 110: 13961-13964.
2. Punjabi NH, Taylor WR, Murphy GS, Purwaningsih S, Picarima H, et al. (2012) Etiology of acute, non-malaria, febrile illnesses in Jayapura, northeastern Papua, Indonesia. Am J Trop Med Hyg 86: 46-51.
3. Kosasih H, Ibrahim IN, Wicaksana R, Alisjahbana B, Hoo Y, et al. (2011) Evidence of human hantavirus infection and zoonotic investigation of hantavirus prevalence in rodents in western Java, Indonesia. Vector Borne Zoonotic Dis 11: 709-713.
4. Lanciotti RS (2003) Molecular amplification assays for the detection of flaviviruses. Adv Virus Res 61: 67-99.
5. Smithburn K, Hughes T, Burke A, Paul J (1940) A neurotropic virus isolated from the blood of a native of Uganda. The American Journal of Tropical Medicine and Hygiene 1: 471-492.
6. Mackenzie JS, Williams DT (2009) The zoonotic flaviviruses of southern, south-eastern and eastern Asia, and Australasia: the potential for emergent viruses. Zoonoses Public Health 56: 338-356.
7. Chaintoutis SC, Chaskopoulou A, Chassalevris T, Koehler PG, Papanastassopoulou M, et al. (2013) West Nile virus lineage 2 strain in Greece, 2012. Emerg Infect Dis 19: 827-829.
8. Bakonyi T, Ferenczi E, Erdelyi K, Kutasi O, Csorgo T, et al. (2013) Explosive spread of a neuroinvasive lineage 2 West Nile virus in Central Europe, 2008/2009. Vet Microbiol 165: 61-70.
9. Zeller HG, Schuffenecker I (2004) West Nile virus: an overview of its spread in Europe and the Mediterranean basin in contrast to its spread in the Americas. Eur J Clin Microbiol Infect Dis 23: 147-156.
10. Monini M, Falcone E, Busani L, Romi R, Ruggeri FM (2010) West nile virus: characteristics of an african virus adapting to the third millennium world. Open Virol J 4: 42-51.
11. Sambri V, Capobianchi M, Charrel R, Fyodorova M, Gaibani P, et al. (2013) West Nile virus in Europe: emergence, epidemiology, diagnosis, treatment, and prevention. Clin Microbiol Infect 19: 699-704.
12. Colpitts TM, Conway MJ, Montgomery RR, Fikrig E (2012) West Nile Virus: biology, transmission, and human infection. Clin Microbiol Rev 25: 635-648.
13. Olson JG, Ksiazek TG, Gubler DJ, Lubis SI, Simanjuntak G, et al. (1983) A survey for arboviral antibodies in sera of humans and animals in Lombok, Republic of Indonesia.

Ann Trop Med Parasitol 77: 131-137.

14. Lindsey NP, Staples JE, Lehman JA, Fischer M, Centers for Disease C, et al. (2010) Surveillance for human West Nile virus disease - United States, 1999-2008. MMWR Surveill Summ 59: 1-17.

# Chapter 11

## Enhancing knowledge and awareness of dengue during a prospective study of dengue fever

*Southeast Asian J Tropical Medicine Public Health, 2004*

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## ABSTRACT

In 1992, the Indonesian CDC implemented strategies to control and prevent dengue fever (DF) by including community involvement to reduce larva breeding sites and a mass health education program. To contribute to this effort, we incorporated an educational component into a prospective study of DF conducted at two textile factories in Bandung. This education provided: a lecture on the signs and symptoms of dengue and ways to prevent the disease, posters in the health clinic at each factory and handouts given to each volunteer with an explanation of symptoms. Upon enrollment, each participant completed a questionnaire to gather demographic information. Additionally they were given a brief (non-standardized) test (PRE-test) of their dengue knowledge, which was verbally administered by the study physicians. Five questions (15 point system) were designed to assess the participant's ability to recognize and describe aspects of dengue in lay terms. The subject material included: the symptoms of acute DF, transmission of dengue virus, and basic steps for disease prevention. The same questionnaire was re-administered 18 months later (POST-test), and the results were compared. A total of 2,340 participants completed both the PRE- and POST-tests; there were 1,373 males and 967 females, median age 36 years (range 18-59). Only 0.3% of participants scored EXCELLENT (15-14 points) on the PRE-test whereas 8.4% scored EXCELLENT on the POST-test. Fewer participants scored VERY BAD (2-0 points) on the POST-test compared to the PRE-test (1.4% *vs* 4.0%). The average raw scores for the PRE- and POST-tests were 7.8 and 10.1, respectively. Improvement of individual scores correlated highly with educational level. No significant correlation was identified for gender, age, factory location or a diagnosis of dengue during the study. These findings demonstrate that our prospective study enhanced knowledge and awareness of dengue in the volunteers.

## INTRODUCTION

Dengue fever (DF) and dengue hemorrhagic fever (DHF) continue to cause thousands of deaths and morbidity annually. In many Asian countries, severe disease affects children the greatest while milder cases typically occur among

adults. Although dengue illness in adults is often mild or unapparent, the disease burden may significantly impact economic growth and productivity. The average dengue illness may require up to 7-10 days of convalescence prior to the individual's returning to work. Many cases can cause even more prolonged illness requiring several weeks convalescence. During epidemic years, the financial impact can be quite substantial, especially in developing countries such as Indonesia.

The role of community education programs is well recognized by health officials as an important factor in efforts to control and prevent dengue illness. The Indonesian Ministry of Health Communicable Diseases Center (CDC), in conjunction with the WHO, has established a mass health education campaign along with programs to reduce larva-breeding sites. The campaign primarily conveys messages to cover, empty/clean water containers ('*3M's*' in indonesian language) or discard unused containers or other items that may fill with water, which may become selective breeding sites for *Aedes* mosquito larvae. Few evaluations of the impact of health education or community messages have been conducted in Indonesia. Public health education programs in other countries have clearly demonstrated success in increasing awareness of the disease and its transmission [1-3]. To contribute to the Indonesian CDC's program of increasing awareness to combat and prevent dengue infection, we incorporated an educational component into our prospective study of DF and DHF among adults at two textile factories in Bandung, West Java. Although our study budget did not allow for a comprehensive public health educational program, we desired to provide this benefit to the participants while accomplishing the primary objective of improving our understanding of dengue infections among adults. This report briefly summarizes the findings of our efforts.

## MATERIALS AND METHODS

In August 2000, a prospective study of DF and DHF in adults was initiated at two textile factories (Grandtex and Naintex) in Bandung, a large urban city on the island of Java, Indonesia. During enrollment, demographic information was collected from all participants (n=2,536) and a verbal PRE-test (non-standardized)

of dengue knowledge was administered in the Indonesian language (Fig 1). The study investigators designed this brief dengue knowledge assessment test in lay terms consisting of five questions to assess each participant's basic knowledge of symptoms of dengue infection, transmission of dengue virus, and methods for the prevention of dengue illness. An arbitrary scoring system (maximum 15 points) was designed to provide grades as shown in Figure 1. Shortly thereafter, a baseline educational program was developed to provide general dengue information to the cohort. This included a didactic lecture taught by the study physicians, printed informational handouts, and posters printed by the Indonesian CDC which were displayed at or near each factory occupational health clinic throughout the duration of the prospective study. The same verbal dengue knowledge assessment test (POST-test) was re-administered 18 months later (February 2002) and compared with the results of the PRE-test. Data were evaluated descriptively and analyzed using nonparametric statistical analyses.

The Institutional Review Boards of the US NAMRU-2 and the Indonesian National Institute of Health Research and Development approved the prospective study and verbal questionnaires (DoD#30855). All participants granted informed, written consent prior to their enrollment.

Dengue Knowledge

Symptoms (max point 5)

1. What are the symptoms of patients who suffer from dengue hemorrhagic fever?
   - fever — 1 point
   - body aches/headache — 1 point
   - red spot — 1 point
   - bleeding from nose or gum etc — 1 point
   - other symptoms (see below)[a] — 1 point

Transmission (max point 5)

2. how is this disease transmitted?[b]
   1.by *Aedes* mosquito(2) 2.by mosquito(1) 3.No idea(0)
3. when does this mosquito usually bite?
   1.in the morning and afternoon(2) 2.in the morning(1)
   3.in the afternoon(1) 5.at night(2) 4.no idea(0)
4. where is the mosquito's breeding place?
   1.clear water(1) 2.dirty water(0) 3.rice terrace(0) 4.no idea(0)

Prevention (max point 5)

5. what prevention steps need to be taken to aoid this disease?
   - spray the mosquitos (fogging) — 1 point
   - put abate powder in the water — 1 point
   - bury unused cans, etc — 1 point
   - wash water containers — 1 point
   - close water containers — 1 point

| Grade | Score |
|---|---|
| Excellent | 15-14 |
| Very Good | 13-12 |
| Good | 11-9 |
| Fair | 8-6 |
| Bad | 5-3 |
| Very Bad | 2-0 |

[a]acceptable answers : stomachache, fatigue, nausea, vomiting, shock

[b]only answer # 1 or 2 is correct

**Figure 1.** Dengue knowledge assessment test

## RESULTS

A total of 2,340 participants in the cohort completed both the PRE and POST dengue knowledge assessment tests. There were 1,373 males and 967 females at the two factories combined; the mean age of workers was 36.2 years (range 18-59). A summary of responses is shown in Fig 2. The average raw scores for the PRE and POST-tests were 7.8 and 10.1, respectively.

The majority scored FAIR (39.9%) or GOOD (38.4%) on the PRE-tests. However, the average grade shifted to GOOD (42.5%) or VERY GOOD (23.8%) for the POST-testing. Only 0.3% of participants scored EXCELLENT (15-14 points) on the

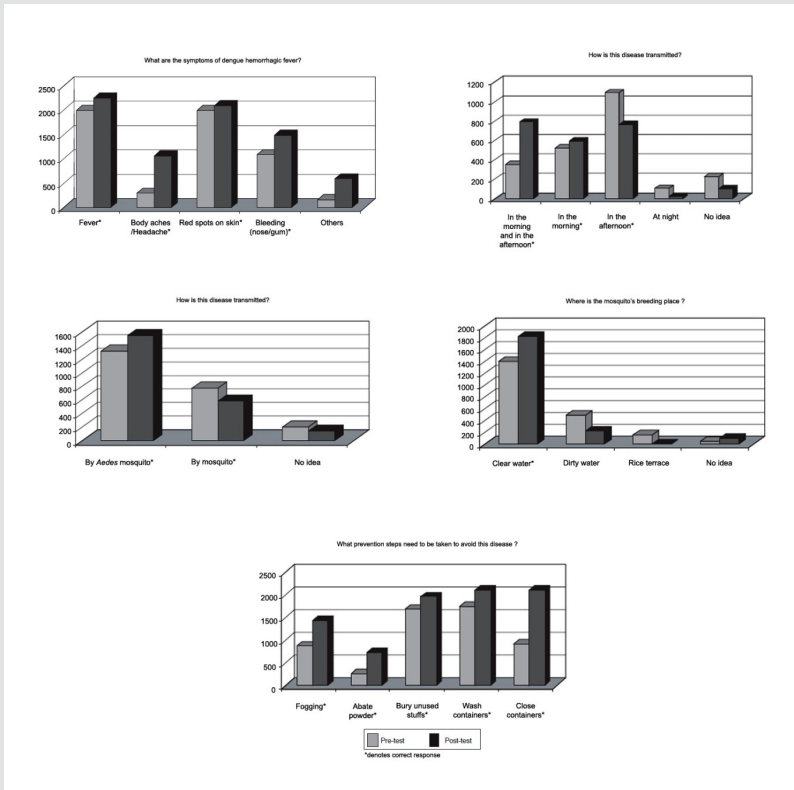


**Figure 2.** Results of dengue knowledge assessment test.

PRE-test whereas 8.4% scored EXCELLENT on the POST-test. Fewer participants scored VERY BAD (2-0 points) on the POST-test compared to the PRE-test (1.4% *vs* 4.0%). Improvement of individual scores correlated highly with educational level (Pearson rank correlation=0.389, significant at 0.01 level, 2-tailed). Factory workers with college education scored higher on average than those with high school, secondary and primary school education (Table 1). No significant correlation was identified for gender, age, factory location or a diagnosis of dengue during the prospective study.

**Table 1.** Comparison of dengue knowledge assessment test scores (pre and post) versus educational level.

| | Mean raw scores | | p-value |
|---|---|---|---|
| | PRE-test | POST-test | |
| Primary | 6.27 | 8.90 | <0.01 |
| Secondary | 7.38 | 9.67 | |
| High | 8.30 | 10.51 | |
| College | 8.96 | 11.35 | |
| Total | 7.77 | 10.08 | |

In question-specific analysis, interesting trends were found. At PRE- and POST-testing, most workers knew that fever and petechiae were symptoms of dengue illness (85.4%, 96.2%, respectively), but very few 12.6% (295) related body aches/headache as a symptom on the PREtest compared to 45.6% (1,066) on the POST-test. Bleeding from the nose or gums was also not a well-known symptom at baseline (46.6%). During both the PRE/POST-tests, the preferred answer for the method of disease transmission was by '*Aedes* mosquito' versus 'mosquito'. Only 4.6% (108) answered incorrectly that *Aedes aegypti* bite at night while most (78%, 1,831) answered correctly that the mosquito's breeding place is in clear water. The final question relating to vector control measures included five theoretically correct sub-answers of which three are contained in the *3M* educational messages: bury, wash, and close containers. Responses were evenly distributed among these three answers. Surprisingly at PRE-testing, the use of Abate® powder and fogging were not thought to be adequate prevention techniques even though many communities are made aware of the use of these methods to control outbreaks via the publicity it raises. These findings demonstrate that our prospective study enhanced knowledge and awareness of dengue in the study participants.

## DISCUSSION

The educational program provided during our prospective study appeared to enhance the knowledge and awareness of dengue among the study participants, as assessed by our verbal knowledge test. The most notable finding was that test performance correlated highly with educational level, albeit there was significant POST test improvement at all educational levels. No significant correlation was identified between test scores and age, factory location or gender.

In a prior study, gender and age differences had been recognized as a determinant factor on knowledge of DF and DHF, for example housewives tended to have a better baseline dengue knowledge and elderly individuals tended to be less knowledgeable [4]. Perhaps this was not observed in our cohort since the women surveyed were in the workforce versus being homemakers, and the extreme elderly were not surveyed (maximum age, 59 years). Admittedly, our assessment methods may have been limited and were not intended to provide a stringent assessment of the Indonesia CDC's overall mass health education program for the country. However, our cohort of textile workers provides a representative sampling of a segment of the population in the city of Bandung. Baseline educational skills and targeted health education should be considered key factors in developing knowledge and awareness of dengue and possibly other vector-borne diseases.

## ACKNOWLEDGEMENTS

The authors wish to thank Haditya L Mukri for data analysis, Dr Timothy Burgess and Mr Richard Aiken for editorial assistance on this manuscript. This work was supported by the US Naval Medical Research Center, Silver Spring, Maryland, work unit 61102A.S13.S.D0025.W2025. The opinions and assertions herein are not to be considered as official or as reflecting the views of the US Navy or the Naval service at large.

## References

1. Lloyd LS, Winch P, Ortega-Canto J, Kendall C (1992) Results of a community-based Aedes aegypti control program in Merida, Yucatan, Mexico. Am J Trop Med Hyg 46: 635-642.
2. Espinoza-Gomez F, Hernandez-Suarez CM, Coll-Cardenas R (2002) Educational campaign versus malathion spraying for the control of Aedes aegypti in Colima, Mexico. J Epidemiol Community Health 56: 148-152.
3. Madeira NG, Macharelli CA, Pedras JF, Delfino MC (2002) Education in primary school as a strategy to control dengue. Revista da Sociedade Brasileira de Medicina Tropical 35: 221-226.
4. Van Benthem B, Khantikul N, Panart K, Kessels P, Somboon P, et al. (2002) Knowledge and use of prevention measures related to dengue in northern Thailand. Tropical Medicine & International Health 7: 993-1000.

# Chapter 12

## Early detection of dengue infections using cluster sampling around index cases

*Am J Tropical Medicine Hygiene, 2005*

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## ABSTRACT

A two-year study using a cluster investigation method was conducted in West Jakarta, Indonesia to demonstrate the detection of dengue cases prior to onset of clinical illness. The clusters consisted of family members and neighbors of 53 hospitalized dengue index cases. Among 785 adult and child volunteers enrolled, 17(2.2%) post enrollment dengue (PED) infections were identified. Eight PED cases were asymptomatic and nine were symptomatic. Symptomatic cases included eight with dengue fever and one with dengue hemorrhagic fever (DHF) (grade II). Among the eight asymptomatic PED cases, viremia was detected in two. Eleven volunteers had acute dengue infections at the time of enrollment. Four of the 11 developed DHF, resulting in a total of five DHF cases detected during the investigation. This study design can serve as a benchmark for future investigations that seek to define early immunologic events following dengue infections that contribute to the development of DHF.

## INTRODUCTION

Infection with any of the four serotypes of dengue virus (DEN-1, DEN-2, DEN-3, and DEN-4) can be asymptomatic, result in a mild-to-moderate febrile illness termed dengue fever (DF), or a more severe illness characterized by bleeding and shock called dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) [1,2]. After an initial infection with dengue virus, homologous serotype immunity persists for many years; however, individuals remain susceptible to infection with the remaining dengue virus serotypes. In several studies, DHF occurs 15–80 times more frequently in individuals experiencing a second dengue virus infection compared with individuals experiencing their first infection [3]. The biologic events that lead to a higher frequency of DHF in secondary dengue virus infections compared with primary infections are not completely known.

Antibody-dependent enhancement of dengue virus infection leading to increased viral load is a leading theory for severe diseases caused by dengue virus. Increased levels of circulating dengue virus have been documented in DHF cases compared with DF cases [4-6]. However, the immunologic events that

occur in the early stages of infection that lead to severe disease have not been characterized in patients. In the absence of an animal model for DHF, researchers are forced to try to characterize early immunologic events in patients with recently acquired dengue virus infections. A major limiting factor in such an investigation is identifying individuals prior to the onset of their clinical illness so that early events can be characterized. The current study was designed to overcome that problem by identifying individuals very early in the infection. Family members and nearest neighbors of index cases hospitalized with acute dengue virus infections were enrolled and followed longitudinally to determine the incidence rate of dengue virus infection. This proof-of-concept study was conducted to evaluate the hypothesis that monitoring persons clustered around an index case would be an efficient and practical way to obtain blood samples before, during, and after dengue infection.

## MATERIALS AND METHODS

### Study location and population.

West Jakarta (West Java), Indonesia, was selected as the location to pilot the concept of a dengue cluster study for several reasons. The catchment area for cases is in a tropical, urban setting with approximately 2.5 million inhabitants of various socioeconomic strata, mostly of low income. On average, 5–10 persons may live in a single housing unit measuring $10 \times 15$ meters$^2$. Most neighborhoods are densely populated, with dwellings in relatively close proximity to one another (approximately 10 meters apart) and constructed of cement and/or wood. The average house does not have piped water and some communities share toilet facilities. The government supplies potable water in large cans and water for bathing is often stored in a traditional uncovered, tub-like area (bak mandi) located in bathrooms. The bak mandi has long been a potential source of *Aedes* mosquito larvae,[7] in addition to other open containers used for water storage and various other miscellaneous items (i.e., flower pots, tires) containing water found in close proximity to human dwellings [8]. *Aedes aegypti* mosquitoes are ubiquitous throughout Indonesia and transmission of all four dengue virus serotypes is known to occur. According to the Indonesian Communicable

Disease Control, the three-year incidence of dengue disease in Jakarta was 75.4, 52.4, and 108.5 cases per 100,000 population from 2001 to August 2003, respectively (unpublished data).

**Dengue virus laboratory tests.**

Serum samples were tested for the presence of IgM antibodies to dengue virus using commercial enzyme-linked immunosorbent assay (ELISA) kits (Focus Technologies, Cypress, CA). Hemagglutination inhibition (HI) assays and plaque reduction neutralization tests (PRNTs) were also performed to confirm dengue virus infection and to classify the infection as primary or secondary based on the antibody response [9]. Dengue virus RNA in blood was detected using a reverse transcriptase–polymerase chain reaction (RT-PCR) assay [10]. Blood samples collected from symptomatic volunteers during the acute stage of febrile illness and from suspected asymptomatically infected volunteers were tested for virus by culture in C6/36 cells, and virus was identified using serotype-specific monoclonal antibodies for dengue virus [11].

**Study Design**

Each cluster of volunteers was identified by an index case from the pediatric ward at Sumber Waras Hospital in West Jakarta. Physicians at this local community hospital have a well-established relationship with the surrounding community and are experienced in clinically diagnosing dengue virus infections. For the index cases, children 4–14 years of age were recruited. Ward clinicians were asked to select 1–2 cases per week based on clinical manifestations, together with the detection of IgM antibody to dengue virus by ELISA and/or dengue viral RNA by RT-PCR. In most cases, these diagnostic assays were performed within 48 hours of index case identification. Other considerations in selecting index cases included automobile accessibility to their community and the willingness of the family to participate.

Usually within 48 hours of index case identification, family members and nearest neighbors greater than four years old who lived within a 10-meter radius of the

index case's home were invited to participate. Volunteers who were afflicted with or who had a history of severe anemia, bleeding disorder, or any known immunologic disorder were excluded from the study. On enrollment, we measured the otic temperature, and collected blood samples and demographic data from each study volunteer. Volunteers were followed for 14 consecutive days for fever and other clinical manifestations suggestive of acute dengue virus infection. During the monitoring period, we collected additional blood samples from each volunteer every 2–3 days. We also collected blood specimens from each volunteer who developed a fever or dengue-like signs or symptoms and processed them for a diagnosis of dengue. To confirm that dengue virus was the cause of fever, we tested samples within 24–48 hours of collection for dengue viral RNA by RT-PCR and for IgM antibody to dengue virus by ELISA. If a sample collected from a febrile volunteer was positive for IgM antibodies to dengue virus, the enrollment blood sample for that volunteer was tested for IgM antibodies to dengue virus to determine if seroconversion had occurred. These laboratory tests were performed at the Virology Laboratory of the U.S. Naval Medical Research Unit No. 2. Blood samples treated with EDTA were also obtained at the onset of fever and sent to Sumber Waras Hospital for hematocrit and platelet count determinations.

Febrile volunteers with a positive IgM ELISA or RT-PCR result were encouraged to be hospitalized for close monitoring and serial laboratory tests. Volunteers refusing hospitalization were monitored closely as outpatients until they were afebrile for two consecutive days. Convalescent blood samples were obtained two weeks later and again at six months.

Hospitalized volunteers were bled daily until two days after defervescence, two weeks later, and approximately six months after discharge from the hospital. Blood samples were routinely tested for hemoglobin, hematocrit, platelets, white blood cells, protein, and albumin. A tourniquet test was done on admission and daily ultrasound examinations for ascites and pleural effusions were performed until two days after defervescence to detect evidence of plasma leakage. We classified hospitalized volunteers as DF or DHF (World Health Organization, 1997) [12] following evaluation of the clinical data.

We categorized all volunteers clustered around the index cases into one of three groups: non-dengue (ND) infection, dengue infection at enrollment (ED), or post-enrollment dengue (PED) infection. A PED case was defined as a volunteer who developed fever after enrollment and demonstrated seroconversion for IgM antibodies to dengue virus and/or dengue viremia by RT-PCR or virus isolation. Volunteers who developed fever but were negative for IgM antibodies to dengue virus or viremia were later classified as PED infections if there was a four-fold increase in the HI antibody titer to dengue virus between enrollment and the two-week post-enrollment blood samples. For equivocal HI results (less than a four-fold increase in titer), pre-enrollment and two-week post-enrollment $PRNT_{50}$ titers were compared for a ≥ fourfold increase against one or more dengue virus serotypes. For volunteers who never developed fever, laboratory evaluation of serum samples (dengue serology and virus detection) was still conducted throughout the 14-day monitoring period. Asymptomatic volunteers who were positive for viremia and/or showed a four-fold increase in HI titer between the enrollment and two-week post-enrollment blood samples were also classified as PED cases.

Volunteers that demonstrated evidence of infection (IgM, RT-PCR, or virus isolation), with or without symptoms at the time of enrollment were classified as ED cases. All other volunteers were classified as ND cases.

The U.S. Naval Medical Research Unit No. 2 Institutional Review Board and the Indonesian Ministry of Health Ethical Review Committee reviewed and approved the human use protocol (DoD# 30861) used in the study, in compliance with all U.S. federal regulations governing the protection of human subjects. Informed written consent was obtained from all participants, and if < 15 years of age, from a parent or legal guardian.

## RESULTS

Over a two-year period from October 2001 to October 2003, 53 confirmed dengue index cases were identified in the pediatrics ward at Sumber Waras

Hospital (Table 1 and Figure 1). Infections by all dengue virus serotypes were identified among the index cases. The predominant serotype identified was DEN-1 (10), followed by DEN-2 (8), DEN-3 (5), and DEN-4 (2). The serotype was not determined in 28 index cases. None of the index dengue infections was fatal.

From these 53 dengue index cases, 53 cluster investigations were performed where family members and nearest neighbors surrounding the residence of each index case were monitored for dengue virus infection. A total of 785 volunteers were recruited from the 53 community clusters, of which 541 completed the entire 14-day monitoring period. The 244 volunteers who did not complete the entire monitoring period were included in the analysis since at least one blood specimen was available for evaluation from each volunteer. Table 1 shows the demographics of the volunteers in the study.

Among the 785 cluster volunteers, 175 (22.3%) were classified as ED cases. Table 1 also shows the demographics of ED cases. Eleven ED cases were found to be viremic for dengue at enrollment by the RT-PCR. The DEN-1 serotype was identified in three cases and DEN-2 in eight cases. Confirmatory virus isolation was accomplished in 5 of the 11 cases. The remaining 164 ED cases had evidence of recent dengue virus infection, as indicated by IgM antibodies to dengue virus at enrollment and were classified as convalescent infections (Figure 1).

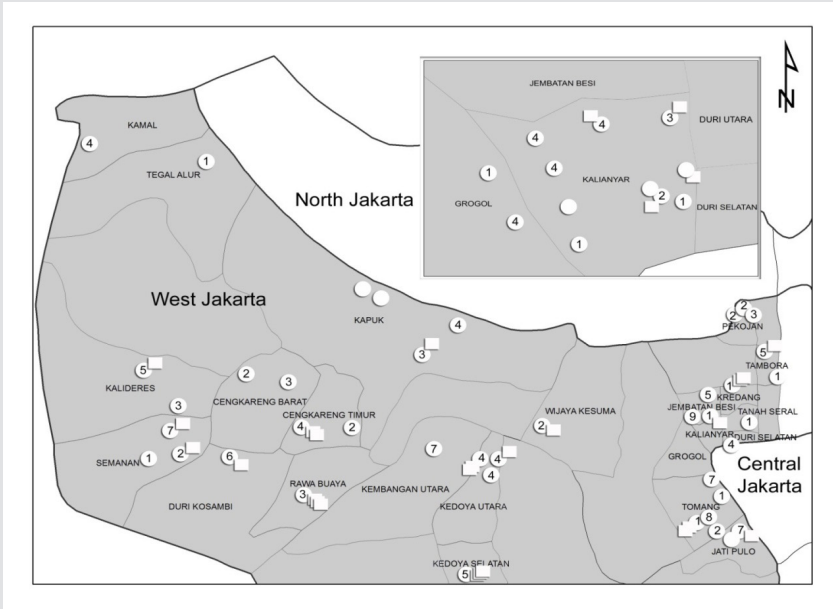


**Figure 1.** Map of index cases and corresponding dengue infections identified in respective clusters in West Jakarta, Indonesia. Each circle represents an index case. Each square represents an acute dengue case identified at enrollment by detection of virus (ED acute infection) or during a two-week period of monitoring by detection of virus or by seroconversion (post-enrollment dengue infection). The numbers inside the circles represent convalescent dengue infections detected by the presence of IgM antibody only at enrollment (ED convalescent infection). The study hospital (Sumber Waras Hospital) is indicated.

Nine of the 11 viremic ED cases had fever at enrollment. The nine febrile volunteers were hospitalized for close monitoring within 24–48 hours of enrollment after dengue serologic and/or RT-PCR results were known. Four of the febrile hospitalized ED cases were subsequently diagnosed with DHF (three grade I and one grade II). All four DHF cases were caused by a DEN-2 virus. Analysis of HI and PRNT titers suggested that two of the DHF cases were secondary infections and two were primary infections. The volunteers with primary infections were 10 and 8 years old, whereas those with the secondary infections were 20 and 21 years old.

Seventeen (2.2%) volunteers were diagnosed as PED cases during the 14-day monitoring period (Table 2), and most (15 of 17, 88%) were among nearest neighbors (Table 1).

**Table 1.** Demographics of volunteers and corresponding dengue infections identified in West Jakata, Indonesia*

| | Number of Index cases | Community volunteers | ED cases | PED cases |
|---|---|---|---|---|
| Total | 53 | 785 | 175 | 17 |
| Males:females | 23:30 | 317:468 | 84:91 | 8:9 |
| Adults | NA | 453 | 87 | 8 |
| Children (4-14 years old) | 53 | 332 | 88 | 9 |
| Mean age (years) | 6.6 | 25.57 | 19.2 | 20.2 |
| Family members | NA | 164 | 32 | 2 |
| Nearest neighbors | NA | 621 | 143 | 15 |

*ED = enrollment dengue cases; PED = post-enrollment dengue cases; NA = Not Applicable

Of the 17 PED cases, 10 were positive by the RT-PCR (1 DEN-1, 7 DEN-2, 1 DEN-3, and 1 DEN-4), and the serotype was confirmed by virus isolation in 5 cases. The other seven PED cases became positive for IgM antibodies to dengue virus during monitoring, and dengue infections were also evident by serocoversion ≥ 4-fold increase in antibody titer) by HI or PRNT. Four of the 17 PED cases were primary infections based on HI and $PRNT_{50}$ results, and all others were secondary infections.

Nine of the 17 PED cases experienced symptomatic illnesses. Eight of the nine were DF and one was diagnosed as DHF grade II. The HI and PRNT patterns comparing preillness and convalescent blood samples suggested a secondary infection in all but three cases (Table 2).

**Table 2.** Characteristics of 17 post-enrollment dengue (PED) infection cases identified

| Study No. | Age (years)/sex | DEN Serotype | Clinical Classification* | Serotype response |
|---|---|---|---|---|
| CL 0102 | 9/F | 4† | DF | Secondary |
| CL 0118 | 18/M | | Asymptomatic | Secondary |
| CL 0417 | 37/F | | Asymptomatic | Secondary |
| CL 0710 | 14/F | | Asymptomatic | Secondary |
| CL 1002 | 7/M | 2 | DF | Primary |
| CL 1211 | 5/M | | DF | Secondary |
| CL 1514 | 30/F | 2 | DF | Secondary |
| CL 1806 | 10/F | 2† | DHF grade II | Secondary |
| CL 2202 | 8/M | 2† | DF | Primary |
| CL 2204 | 40/F | 2† | Asymptomatic | Secondary |
| CL 2408 | 47/F | 1 | Asymptomatic | Secondary |
| CL 3213 | 24/M | 2† | DF | Primary |
| CL 3716 | 9/M | | Asymptomatic | Secondary |
| CL 3911 | 32/F | 2 | DF | Secondary |
| CL 4202 | 11/M | 3 | DF | Secondary |
| CL 5115 | 36/M | | Asymptomatic | Secondary |
| CL 5304 | 7/F | | Asymptomatic | Primary |

* DF = dengue fever; DHF = dengue hemorrhagic fever.

† Positive by both dengue virus reverse transcriptase–polymerase chain reaction and virus isolation in C3/36 cells.

The one PED DHF case was hospitalized five days after enrollment with a fever of 38.8°C and a positive tourniquet test result, along with a history of epistaxis prior to admission that continued for two days while hospitalized. Small pleural effusions and ascites developed on day three, but resolved by day five. The platelet count was normal on admission but decreased to 40,000/$mm^3$ by hospital day six. From admission, total protein and albumin levels decreased from 8.1 g/dL to 5.2 g/dL and from 3.5 g/dL to 2.9 g/dL, respectively (normal levels: protein = 6.4–8.7 g/dL and albumin = 3.5–5.2 g/dL) on day six. The patient remained hemodynamically stable throughout hospitalization and was discharged on day 10.

Asymptomatic dengue virus infections occurred in eight of the PED cases (Table 2). Dengue viremia was detected in two of the eight cases, with DEN-1 identified in one case by RT-PCR and DEN-2 identified in the second case by both RT-PCR and virus isolation. In the case with DEN-1, virus was detected in the blood sample obtained on day 10. For the case with DEN-2, virus was detected in the blood sample obtained on day 4. To our knowledge, these cases, together with the two asymptomatic ED viremic cases mentioned earlier, represent the first documentation of asymptomatic dengue viremia in human volunteers from naturally acquired infections.

Table 3 shows the serotype-specific relationship of dengue viruses identified from index and cluster cases. There were seven instances in which the virus was identified from both the cluster case and the corresponding index case (four ED cases and three PED cases). The infecting serotype of the cluster case corresponded with the infecting serotype of the index case in four instances (all DEN-2) and in three instances the infecting serotypes were different. We did not detect a specific pattern of the infecting serotype between the ED and PED cases.

**Table 3.** Summary of known dengue virus (DEN) serotypes for the index case and corresponding cluster dengue cases (11 viremic ED cases and 17 PED cases)*

| Cluster case ID no. | Cluster case serotype† | Index case serotype |
|---|---|---|
| ED cases | | |
| CL 0602 | DEN-2 | DEN-1 |
| CL 0906 | DEN-2 | DEN-2 |
| CL 1001 | DEN-2 | DEN-3 |
| CL 1101 | DEN-2 | DEN-2 |
| CL 1105 | DEN-2 | - |
| CL 1108 | DEN-2 | - |
| CL 1201 | DEN-2 | - |
| CL 3701 | DEN-1 | - |
| CL 3702 | DEN-1 | - |
| CL 3815 | DEN-2 | - |
| CL 4007 | DEN-1 | - |
| PED cases | | |
| CL 0102 | DEN-4 | - |
| CL 0118 | - | - |
| CL 0417 | - | - |
| CL 0710 | - | DEN-2 |
| CL 1002 | DEN-2 | DEN-1 |
| CL 1211 | - | - |
| CL 1514 | DEN-2 | - |
| CL 1806 | DEN-2 | DEN-2 |
| CL 2202 | DEN-2 | - |
| CL 2204 | DEN-2 | - |
| CL 2408 | DEN-1 | - |
| CL 3213 | DEN-2 | DEN-2 |
| CL 3716 | - | - |
| CL 3911 | DEN-2 | - |
| CL 4202 | DEN-3 | - |
| CL 5115 | - | - |
| CL 5304 | - | DEN-4 |

* ED = enrollment dengue; PED = post-enrollment dengue; ID no. = subject identification number; – = negative result.

† Serotype identified by dengue virus reverse transcirptase–polymerase chain reaction and/or virus isolation.

## DISCUSSION

Our cluster investigation method was designed as an alternative approach to the commonly used prospective cohort study method for investigating the natural history of dengue virus infection. Unlike the cohort study, the cluster investigation method allows the collection of clinical information and biologic samples during the early pre-infection stage. This allows for more precise characterization of the early immunopathologic events that contribute to either mild or severe clinical manifestations of dengue virus infection.

Among 785 volunteers in this investigation, we observed 17 PED (new) dengue infections that were both symptomatic and asymptomatic, including one case of DHF. The calculated incidence rate of dengue infection was 567 cases per 1,000 person-years of follow-up. Based on the one observed DHF case, the calculated DHF incidence rate was 33 DHF cases per 1,000 person-years of follow-up, resulting in a DHF to DF incidence rate ratio of 1:18. This ratio closely approximates other epidemiologic observations, showing that DHF occurs in roughly 5% of dengue virus infections in areas where all four serotypes of dengue virus are endemic [13-15].

We detected 175 individuals who had either a recent dengue virus infection or who were acutely infected at the time of enrollment and diagnosed with DHF. Eleven of the 175 volunteers were found to be viremic. Combining these 11 cases with the PED cases, a total of 28 acutely infected dengue cases were observed among the volunteers (Figure 1), resulting in a calculated incidence of 933 acute dengue cases per 1,000 person-years of follow-up. Of the 28 acute dengue cases, five were classified as DHF resulting in an overall DHF incidence of 166 cases per 1,000 person-years.

Virologic analysis of dengue cases confirmed that all four serotypes circulated in West Jakarta. The DEN-1 serotype was the predominant virus identified among the index cases, but DEN-2 caused most of the cluster cases, including all five DHF cases. We plan to genetically characterize these viruses and compare them to other circulating Asian strains. Using banked sera, we will also examine

the kinetics of infection in both symptomatic and asymptomatic cases by a quantitative RT-PCR. Although an association between the level of dengue viremia and severity of symptomatic disease was previously established, these analyses may provide some insight into whether there is a correlation between the level of viremia and symptomatic or asymptomatic dengue virus infection.

An attractive feature of the cluster investigation method is that it does not rely on outbreak events to accumulate a sizeable number of cases. When longitudinally following a randomly selected cohort, the study population may or may not experience outbreak-related dengue virus transmission, so that to arrive at a sufficient number of DHF and DF cases for statistical analysis, one may have to follow a particular cohort for many months to years. With the cluster investigation method that selects index cases as a reference point, only days to weeks of follow-up are required to define past and present virus activity in an area. A shorter follow-up period would significantly reduce cost and personnel needs.

Cohort studies to date have been unsuccessful in documenting asymptomatic viremia in humans from naturally acquired dengue infection [16]. Given the natural history of dengue virus infection, such silent viremia would seem plausible, but with the design of typical longitudinal cohort studies, infected volunteers are only identified upon the development of symptoms. Asymptomatic infections are only detected by seroconversion of blood samples collected at pre-determined time points. Using a cluster design approach, we were able to document eight asymptomatic dengue infections, two of which demonstrated viremia. To our knowledge, this is the first documentation of asymptomatic viremia in naturally acquired human dengue infections.

The one obvious disadvantage of the cluster method is that volunteers are required to participate in an intensive period of surveillance where, despite being healthy at the time, they are asked to donate blood samples every 2–3 days for a period of two weeks. We had little difficulty recruiting the requisite 10–15 people per cluster and often had to refuse additional enrollment of willing volunteers. With augmented resources and experience, we plan to expand our study design

to new areas (East Jakarta and another major city in West Java, Indonesia) and to increase the number of index cases (6–8 per week) and persons recruited per cluster (up to 20). The major challenge to our cluster study design is maintaining adequate support (finances and trained personnel) for the intensive community monitoring. We believe that the yield of the cluster investigation method is equal to or substantially greater than that of other prospective study methods used to examine the epidemiology of naturally acquired dengue virus infection. As this method is applied to other countries with varying cultural sensitivities and expectations, differing levels of success may be encountered.

## ACKNOWLEDGMENTS

We thank the nurses and physicians in the Internal Medicine Department at Sumber Waras Hospital (Soesilowati Soerachmad, Hansa Wulur, and Fajar Kurniawan Frans J. V. Pangalila); and the Viral Diseases laboratory staff (Sri Hartati and Dasep Purwaganda) for conducting the serologic testing; Yurike S. Tobing and Akterono Dwi for conducting tissue culture and virus isolation; Gustiani and Ungke Anton Jaya for molecular testing; Agus Rachmat for assistance with geographic information system mapping; and Andy Whitehurst for editorial assistance with the manuscript. Financial support: This work was supported by the U.S. Naval Medical Research Center (Silver Spring, MD) work unit 63002A810SD0012 and the Military Infectious Diseases Research Program. Disclaimer: The opinions and assertions herein are not considered as official or views of the U.S. Navy or the Naval Service at large. Disclosure: None of the authors have a financial or personal conflict of interest related to this study.

## References


1. Halstead SB, Deen J (2002) The future of dengue vaccines. Lancet 360: 1243-1245.
2. Endy TP, Chunsuttiwat S, Nisalak A, Libraty DH, Green S, et al. (2002) Epidemiology of inapparent and symptomatic acute dengue virus infection: a prospective study of primary school children in Kamphaeng Phet, Thailand. Am J Epidemiol 156: 40-51.
3. Rothman AL (2003) Immunology and immunopathogenesis of dengue disease. Adv Virus Res 60: 397-419.
4. Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, et al. (2000) Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. J Infect Dis 181: 2-9.
5. Libraty DH, Endy TP, Houng HS, Green S, Kalayanarooj S, et al. (2002) Differing influences of virus burden and immune activation on disease severity in secondary dengue-3 virus infections. J Infect Dis 185: 1213-1221.
6. Wang WK, Chao DY, Kao CL, Wu HC, Liu YC, et al. (2003) High levels of plasma dengue viral load during defervescence in patients with dengue hemorrhagic fever: implications for pathogenesis. Virology 305: 330-338.
7. Jumali, Sunarto, Gubler DJ, Nalim S, Eram S, et al. (1979) Epidemic dengue hemorrhagic fever in rural Indonesia. III. Entomological studies. Am J Trop Med Hyg 28: 717-724.
8. World Health Organization (2000) Dengue/Dengue Haemorrhagic Fever Prevention and Control Programme in Indonesia. Geneva: World Health Organization Report of an External Review Jakarta World Health Organization SEA-Haemorrhagic Fever 73.
9. Russell PK, Udomsakdi S, Halstead SB (1967) Antibody response in dengue and dengue hemorrhagic fever. Jpn J Med Sci Biol 20 Suppl: 103-108.
10. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV (1992) Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J Clin Microbiol 30: 545-551.
11. Tesh RB (1979) A method for the isolation and identification of dengue viruses, using mosquito cell cultures. Am J Trop Med Hyg 28: 1053-1059.
12. World Health Organization (1997) Dengue Haemorrhagic Fever: Diagnosis, Treatment Prevention and Control. Second edition Geneva: World Health Organization.
13. Halstead SB (1997) Epidemiology of dengue and dengue hemorrhagic fever. Gubler DJ, Kuno G, eds. Dengue and Dengue Hemorrhagic Fever New York: CAB International: 23-24.
14. Kliks SC, Nimmanitya S, Nisalak A, Burke DS (1988) Evidence that maternal dengue antibodies are important in the development of dengue hemorrhagic fever in infants. Am J Trop Med Hyg 38: 411-419.

15. Guzman MG, Kouri G (2002) Dengue: an update. Lancet Infect Dis 2: 33-42.
16. Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, et al. (1997) Dengue in the early febrile phase: viremia and antibody responses. J Infect Dis 176: 322-330.

# Chapter 13

Summary and discussion

## SUMMARY AND DISCUSSION

After sporadic outbreaks were reported during the 18th and 19th centuries, dengue emerged as the most important arbovirus only after severe DHF/DSS occurred in Southeast Asia after the World War II [1]. Since then, the epidemiology, virology, immunology, clinical pathogenesis, treatment and prevention of dengue virus infections have been studied by various researchers on different continents. Nevertheless, many controversies and gaps remain to be elucidated. For instance, the commonly quoted statement "dengue causes 50-100 million cases globally every year" may not reflect the reality but should be regarded as "dengue infections", which include a large proportion of asymptomatic infections [1]. Many more controversies and knowledge gaps still exist such as: the usefulness and limitations of the WHO clinical classifications criteria of the years 1997, 2009 and 2011; insight into the pathogenesis of severe dengue (virulence of dengue virus subtypes, abnormal or accelerated T cell responses, autoimmune phenomena or second infections) [2]; the unavailability of rapid and accurate diagnostic tests [3], animal models [4] and antivirals [5]; and a safe and effective vaccine [6].

To improve our understanding of the epidemiology and pathogenesis of dengue infections, prospective studies which can accurately identify and characterize dengue infections and their severity are needed [7]. Different kinds of prospective studies can be used, including population-based cohort studies or cluster studies in communities, and hospital based studies. The first population-based dengue cohort study was carried out among school children in Rayong, Thailand in 1984, followed by five similar studies carried out in Bangkok, and other cities including Yangon in Myanmar, Yogyakarta in Indonesia and Managua in Nicaragua [7].These cohort studies had several outcomes including a better understanding of disease burden on different populations, including the economic impact and the characterization of the determinants of disease severity. In addition, populations with a well-defined natural history of dengue infections were identified, also providing the field site infrastructure to conduct phase III dengue vaccine efficacy studies [7]. The need for longitudinal cohort studies in adults also became evident as

the number of uncomplicated and complicated dengue infections in adults increased during the last three decades, including during outbreaks [8-10]. In addition, prospective cluster studies within certain communities were carried out in order to analyze transmission among household members, and to be able to identify early and new onset infections that allow identification of clinical and immunologic predictors of severe disease [11,12].

This thesis describes results from several prospective studies carried out in West Java, Indonesia. The first is a prospective cohort study conducted at three textile factories in Bandung from 2000-2004 and 2006-2009, involving 4380 adults who were monitored daily and had blood taken quarterly. The second was a prospective community clusters study in 2001-2003 in West Jakarta, where family members and close neighbors of an index patient were followed for two weeks to collect pre-illness and consecutive specimens as well as signs and symptoms of a possible dengue infection. In addition, this thesis also presents the findings from a large dengue outbreak in 2004, the national influenza surveillance carried out between 2003 and 2007, and a hospital-based hantavirus surveillance study performed between 2004 and 2005. These studies were funded by the United States Naval Medical Research Unit no 2 (US-NAMRU#2), in collaboration with Sumber Waras Hospital, Jakarta, Hasan Sadikin Hospital and Faculty of Medicine Universitas Padjadjaran, Bandung, and the National Institute of Health Research and Development, Ministry of Health, Jakarta, Indonesia.

**Chapter 2** presents the epidemiology, virology and clinical aspects of dengue infections in a cohort of 4380 adult factory workers in Bandung, West Java, who were observed for a period of 79 months. This study demonstrates that dengue contributed to 12.41% of fever cases and that the actual annual dengue incidence rate was 17.3 cases/1000 population per year, 43 times higher than the national reports. This study further reveals that the actual number of dengue infections per population per year should also considered asymptomatic infections which was 2.6x the incidence rate. This new epidemiological data is important for clinicians and health policy makers, as the study demonstrates that it is important to include dengue in the differential diagnosis of undifferentiated febrile illnesses. It is also necessary

to develop strategies to control dengue transmission which is much more frequent than previously known. Furthermore, dengue cases were found during almost the whole year, with the peak during the wet season that generally ranges from December to March. All serotypes were detected as circulating every year, although they were rarely found simultaneously in the same month. This finding demonstrates that Bandung is a hyper-endemic area of dengue infections. From a total of 268 confirmed cases, only one DSS case was diagnosed. The remaining were DHF grade one and grade two, and the mild form, dengue fever with or without hemorrhagic manifestations. Severe cases were associated with DENV-3, which was consistently reported as the main cause of outbreaks in Indonesia. Our data also suggest that asymptomatic cases were associated with DENV-4. In this study, we also found evidence of successive dengue infections in seven patients. This finding confirms that infections with certain serotypes may not protect individuals from infections with other serotypes. Finally, our study has not only provided precious data demonstrating the importance of dengue in West Java but has also characterized the natural course of disease in an adult population, which is a prerequisite for future vaccine studies.

While **chapter 2** provides data on natural dengue infections during endemic conditions, data from a dengue outbreak in 2004, that included 50,000 cases and 603 deaths in Indonesia, is reported in **chapter 3**. The data are important, since periodic outbreaks of dengue have emerged in Indonesia since 1968. The chapter provides epidemiological, clinical and virological data from 10 hospitals in five municipalities in Jakarta, Indonesia. Our investigations confirm that all municipalities were affected by this outbreak, while all four dengue serotypes were simultaneously circulating, with DENV-3 as the most predominant. Genotype analysis reveals that the circulating DENV-3 was identical to that which caused the 1998 epidemic. Simultaneous circulation of four or multiple serotypes with DENV-3 as the most predominant has also been reported in previous dengue outbreaks in Indonesia, including during the largest outbreak in 1998. The circulation of multiple serotypes with DENV-3 predominance - besides the presence of naïve populations and the circulation of novel or more virulent virus strains - might therefore create the conditions for a dengue outbreak to occur in an endemic situation. Our investigation also shows that all age groups were affected and that the proportion of DHF was significantly higher during

an outbreak compared to endemic conditions, despite the possibility of an underestimation of DHF cases due to the lack of serial full blood counts to demonstrate hemoconcentration. Finally, similar to the previous report from Semarang [13], dengue was not confirmed in a large proportion (33%) of cases.

The first two chapters have described West Java as a hyper-endemic region where all four DENV seroytpes were circulating. According to the antibody dependent enhancement theory (ADE), this condition puts the populations at increased risk to develop severe disease in case of secondary infections. In **chapter 4,** evidence is given of three heterologous successive dengue infections in four study participants. The first infection was prior to their enrolment in the study, and two infections were during their participation in this study. These patients, of ages ranging between 29 and 42 years old, did not have (or only had low) titer neutralizing antibodies to the infecting serotypes prior to their secondary infections. Only one of the four secondary infections resulted in a more severe DHF grade II. Prior to the secondary infection, this patient had evidence of a previous DENV-2 infection and a low titer ($PRNT_{50}$ 29) to the infecting serotype (DENV-4). The sequence of infecting serotypes in the other study participants who only experienced dengue fever varied, from DENV-1 to DENV-3, DENV-2 to DENV-3 and DENV-2 to DENV-4. All tertiary infections resulted in a mild form of dengue infection. In two cases, DENV-3 followed a previous DENV-4 infection while in the other two cases, the serotypes of the third infection could not be determined. Pre-illness sera prior to the third infections showed neutralizing antibodies to all serotypes. The intervals between the second and third infections were 5, 11, 18 and 18 months, respectively. A milder form of illness during tertiary infections might correlate with the short duration of viremia because dengue viruses could only be isolated in one of the four cases. Altogether, our study provides evidence that the presence of cross-reactive neutralizing antibodies does not necessarily protect against future dengue infections. On the contrary, as shown in one case, it may lead to a more severe case (DHF).

As primary dengue infection is one of the risk factors for developing severe secondary infections [3], it is important to distinguish between these two types of infections. According to the WHO guidelines [3,14], the hemagglutination

inhibition (HI) test can be applied for this purpose. In **chapter 5**, the performance of HI test in discriminating primary and secondary infection is therefore compared with the IgG ELISA antibody assay using plaque reduction neutralization antibody test (PRNT) as the gold standard. IgG ELISA assay is recommended since results are comparable to PRNT and easier to perform. We found that the HI test can be used, however using modified criteria. According to our results, titers ≤80 in convalescent specimens are indicating primary infections and titers >640 should be considered secondary infections. Titers between 160 and 640 in convalescent specimens are difficult to interpret and other tests such as the IgG ELISA or PRNT should subsequently be used in these cases. In addition, apart from discriminating between primary and secondary infection, the HI test is also recommended to confirm a dengue diagnosis. [3,14]. However, the test is cumbersome to perform, as it may cross-react with other Flaviviruses such as Japanese encephalitis, and only provides retrospective diagnosis.

Hence, better diagnostic tests are needed. The gold standard, virus culture and isolation assay, are technically difficult and time consuming. In addition, molecular assays such as RT-PCR are robust but expensive, and can only be done in large laboratories or research institutes [3]. Finally, serological tests, especially the rapid diagnostic tests are easy to perform, but IgM antibody results can only be interpreted correctly if specimens are taken five days after onset of illness [3]. In secondary cases, paired IgG testing is often required as IgM antibodies may not be detected [3]. Several commercial assays to detect dengue non-structural 1 (NS1) protein have therefore been developed recently. NS1 is encoded by the virus genome, secreted by infected cells in a soluble form, and can be detected in the early stage of the disease several days prior to the appearance of anti-dengue IgM antibodies. The test is relatively inexpensive, easy to perform, and fast. In **chapter 6**, the diagnostic and predictive values of an NS1 antigen assay to early diagnose dengue infections early was evaluated. We found a sensitivity of 46.8%, which was the lowest compared to studies from other countries. However, the sensitivity in primary infection was 100% and the test is therefore useful in travel medicine. The sensitivity also varies across the infecting serotypes, with the highest in DENV-3 (47.1%), followed by DENV-1 (40.9%), DENV-2 (30%) and DENV-4 (27%). Furthermore, we found NS1 is less frequently detected in those patients with previous infections with more than one serotype, while NS1

was more frequently detected in females, those with more severe cases (dengue fever with hemorrhagic manifestations and DHF) and individuals with lower platelet counts (<100,000/mm3).

The importance of an early, rapid, robust and cheap confirmation of the diagnosis dengue is not only important for the management at the individual or community level, but also leads to considerations of alternative diagnosis when dengue cannot be confirmed [3]. **Chapters 7, 8, 9** and **10** discuss four infectious diseases that should be considered by clinicians in West Java in the differential diagnosis of febrile patients when signs and symptoms are undifferentiated. **Chapter 7** reports on chikungunya infections among adult study participants in Bandung. Unlike previous studies where chikungunya is often reported from focal epidemics, our study found continuous chikungunya infections, suggesting that this virus is endemically transmitted in Bandung. Furthermore, chikungunya should be considered in differential diagnosis of undifferentiated febrile illness as the proportion of chikungunya infections among the acute febrile illness cases is high (7.1%), while arthralgia is not a prominent reported symptom. We also identified possible repeated chikungunya infections in three patients, suggesting that either chikungunya antibodies from previous infections might not provide life-long immunity and/or different strains might circulate in Bandung. Finally, we documented the persistence of IgM antibodies and concluded that this phenomenon complicates the diagnosis as paired specimens are needed to observe the increasing titers in convalescent specimens.

**Chapter 8** reports on influenza, including the highly pathogenic influenza A/H5N1, to consider in the differential diagnosis of dengue. Results were collected from a national influenza surveillance program, conducted from 2003 to 2007, which included data from six sites in three cities from West Java. Among patients with influenza like illness, influenza was identified in 20.1% of patients. Unlike in countries with a temperate climate, Influenza viruses circulated throughout the year in Indonesia with the peak during the rainy season from December to March. It is important to distinguish dengue from influenza not only because the treatment is different but also because precautions for the family, school or working place are different. This is particularly important with regard to influenza

A/H5N1, that may have a mortality rate up to 82% [15], but the outcome of which is more favorable when Oseltamivir is administered early [16]. Influenza A/H5N1 was often not considered during the acute febrile illness because alternative diagnoses were made (dengue, typhoid fever, diarrheal disease, respiratory tract infection). In febrile patients with shortness of breath, dengue with pulmonary edema was often mistakenly diagnosed. This national surveillance also documented antigenic drift in circulating influenza A and B virus strains, which were similar to the World Health Organization (WHO) vaccine strains but which were detected much earlier than the date of their designation.

**Chapter 9** documents by means of by clinical, serological and epizoological data, the first Indonesian Seoul virus infection, which belongs to the hantavirus genus. Signs and symptoms, although consistent with hantavirus infection, such as fever, myalgia, bleeding tendency, leucopenia, thrombocytopenia, increased serum transaminases, often resemble severe dengue and the two infectious diseases may be difficult to differentiate. Therefore, hantavirus infection was diagnosed by elevated hantavirus IgM and IgG antibodies, and in the absence of evidence of dengue infection. Furthermore, the diagnosis of hantavirus infections was also supported by the higher prevalence of hantavirus IgG antibodies in rodents trapped in the vicinity of the patient's home, compared with rodents from a control area (13.2% vs. 4.7%, p=0.04) as well as the detection of Seoul virus in the organs of 71% of the seropositive rodents.

**Chapter 10** presents the first evidence of West Nile virus (WNV) in Indonesia. This virus was discovered from archived samples collected during an acute febrile illness study that enrolled patients suspected of hantavirus infection at two hospitals at Bandung, West Java, Indonesia during 2004-2005. The WNV positive sample came from a 15 year old boy admitted for systemic febrile illness with epistaxis, gastrointestinal symptoms, elevated serum transaminases, leucopenia and thrombocytopenia. No neurological symptoms were reported and the patient was discharged after full recovery. Genetic comparisons revealed the closest match with the first WNV strain isolated an isolate from Uganda within lineage 2. This lineage is less virulent than the lineage 1, the common cause of viral encephalitis in the US, and typically associated with large outbreaks.

Apart from dengue virus, the infectious agents described in **chapters 7 to 10** are only four of many other viruses that need to be considered in the differential diagnosis of febrile patients presenting in Indonesia. The development of point of care assays that may differentiate bacterial from viral infections are therefore of great value as it may rationalize prescription of antimicrobial drugs and the management of febrile patients. Furthermore, since dengue is a prevalent illness with a sometimes complicated course, a robust diagnostic point of care assay to confirm or exclude dengue in acute febrile cases is urgently needed.

While the development of a dengue vaccine is challenged by the requirement to provide protection to the four serotypes without exposing the recipients to the risks of ADE, the only preventive community measures that are presently available are eliminating mosquito breeding places, improvement of knowledge to increase awareness, which temporary precautions to take, when to report to the authorities, and what the warning signs are to send a patient to a hospital [3]. Two studies provide valuable information associated with the prevention in communities. The first study aimed to enhance the knowledge and awareness of dengue in adult populations working in two textile factories in Bandung. The second study was a close virological and clinical observation in the communities to anticipate prospective cases after an index case was identified.

**Chapter 11** explores how the incorporation of education into our prospective dengue study affects knowledge about dengue. Education was undertaken in the form of lectures, posters and handouts, describing the signs and symptoms of dengue, the methods of transmission, and how to prevent the disease. Upon enrolment, a questionnaire was verbally administered by the study physicians, exploring participant knowledge regarding dengue. The same questionnaire was re-administered 18 months later. Of the 2340 participants that completed both tests, only 0.3% of participants scored 'excellent' on the pre-test whereas 8.4% scored 'excellent' on the post-test. Fewer participants scored 'very bad' on the post-test compared to the pre-test (1.4% vs. 4.0%). The average raw scores for the pre- and post-tests were 7.8 and 10.1, respectively. Improvement of individual scores correlated highly with educational level. These findings demonstrate that our prospective study enhanced knowledge of dengue in the volunteers.

**Chapter 12** describes two week-observation periods in families of 53 index cases and their close neighbors in West Jakarta in 2002-2003. In total, 785 family members or close neighbors were observed, and 192 (24.5%) dengue infections were documented, consisting of 175 (22.3%) cases prior to or during enrolment and 17 (2.2%) newly developed cases after enrolment. These 17 newly developed dengue cases consisted of eight asymptomatic infections, eight dengue fever infections, and one DHF grade II infection. This study demonstrated that one hospitalized dengue patient can be linked to approximately 3.6 other dengue infections occurring in close proximity of where the index patient is living, suggesting the importance of community involvement to conduct preventive measures and observation. Furthermore, our study showed that asymptomatic cases may also significantly contribute to the spreading of dengue virus since viremia was detected in two out of the eight asymptomatic cases.

## Options for future research

Several research questions remain unanswered also because of the technical limitations of our studies and new findings need confirmation or have generated novel research questions for future studies. A selection of these questions is listed below.

1) *Determination of the causes of undifferentiated fever*:

Approximately 62% of the acute febrile cases from the prospective cohort study (**chapter 1**) and 30% of the fever cases with thrombocytopenia from the hospitalized hantavirus study (**chapter 9**) need further examination to identify etiologies other than dengue. As no similar studies have been conducted previously, further tests will provide clinicians with guidance for differential diagnosis, public health authorities with data about the burden of each disease and valuable information when a novel agent is identified.

2) *Improve the evidence of endemic transmission, mild illness and repeated chikungunya infections*:

Surveillance should be conducted in larger areas involving more clinics and

hospitals to provide better epidemiological, virological and clinical data about chikungunya infections. To confirm the evidence of repeated chikungunya infections in our previous cohort, sequencing analysis should be done to determine different strains or genotypes circulating in West Java and plaque reduction neutralization test to demonstrate the presence of earlier chikungunya infections.

3) *Improve the methods in cluster studies*:

In our study, many dengue infections in family members or close neighbors had already occurred before enrollment. An earlier enrollment of community cases might be achieved when index cases are retrieved from primary health centers instead of hospitals.

4) *Development of biomarkers that identify those at risk for severe disease*.

Dengue is a common cause of febrile illness but fortunately only a minority of patients will develop severe disease. However, so far there are no sensitive and specific biomarkers that can identify those at risk for severe disease. This puts a heavy burden on the health care system, especially during outbreaks. Soluble markers may be identified in pre and early illness serial specimens from asymptomatic, mild or severe dengue infections.

5) *Improve our understanding in the kinetics of neutralizing antibodies*

Neutralizing antibodies as a response to a natural dengue infection with a certain serotype may provide protection from infection with other serotypes until three months post-illness. However, the titer that may provide protection and the titer that may cause ADE leading to severe cases have not been defined [17]. As neutralizing antibodies are also the parameter used to measure the immunogenicity of a vaccine [18], it is important to further study the kinetics of neutralizing antibodies after well-characterized primary, secondary and tertiary natural dengue infections using serial sera, collected on the quarterly basis. Finally, it is also important to evaluate immune response after vaccination

in patients with known primary natural infections.

6) *Determine whether some subjects are protected against dengue infection*:

Our study reveals that a small proportion (3.1%) of adults has never been exposed to dengue infections as indicated by their negative IgG antibodies. As seroconversion is estimated as high as 10% a year [1], it would be interesting to analyze why this adult population remains seronegative.

7) *Evaluation of the various WHO classification systems*:

The controversies regarding the clinical classification of the WHO from 1997 [14], 2009 [3] and WHO 2011 [19] have not yet been resolved [20-24], and a multi-center study using a well-designed protocol should be conducted.

## References


1. Halstead SB (2008) Dengue: overview and history. In: Halstead SB, editor. Dengue. London: Imperial College Press. pp. 1-9.
2. Halstead SB (2008) Pathogenesis: risk factors prior to infection. In: Halstead SB, editor. Dengue. London: Imperial College Press. pp. 219-244.
3. WHO (2009) Dengue guidelines for diagnosis, treatment, prevention and control.
4. Zompi S, Harris E (2012) Animal models of dengue virus infection. Viruses 4: 62-82.
5. Alen MM, Schols D (2012) Dengue virus entry as target for antiviral therapy. J Trop Med 2012: 628475.
6. Halstead SB (2012) Dengue vaccine development: a 75% solution? Lancet 380: 1535-1536.
7. Endy TP, Yoon IK, Mammen MP (2010) Prospective cohort studies of dengue viral transmission and severity of disease. Curr Top Microbiol Immunol 338: 1-13.
8. Guilarde AO, Turchi MD, Siqueira JB, Jr., Feres VC, Rocha B, et al. (2008) Dengue and dengue hemorrhagic fever among adults: clinical outcomes related to viremia, serotypes, and antibody response. J Infect Dis 197: 817-824.
9. Tantawichien T (2012) Dengue fever and dengue haemorrhagic fever in adolescents and adults. Paediatr Int Child Health 32 Suppl 1: 22-27.
10. Cavalcanti LP, Vilar D, Souza-Santos R, Teixeira MG (2011) Change in age pattern of persons with dengue, northeastern Brazil. Emerg Infect Dis 17: 132-134.
11. Likosky WH, Calisher CH, Michelson AL, Correa-Coronas R, Henderson BE, et al. (1973) An epidermiologic study of dengue type 2 in Puerto Rico, 1969. Am J Epidemiol 97: 264-275.
12. Waterman SH, Novak RJ, Sather GE, Bailey RE, Rios I, et al. (1985) Dengue transmission in two Puerto Rican communities in 1982. Am J Trop Med Hyg 34: 625-632.
13. Suharti C, van Gorp EC, Dolmans WM, Groen J, Hadisaputro S, et al. (2009) Hanta virus infection during dengue virus infection outbreak in Indonesia. Acta Med Indones 41: 75-80.
14. WHO (1999) Prevention and control of dengue and dengue hemorrhagic fever- a comprehensive guidelines. New Delhi: WHO Regional Office for South-east Asia
15. Soepandi PZ, Burhan E, Mangunnegoro H, Nawas A, Aditama TY, et al. (2010) Clinical course of avian influenza A(H5N1) in patients at the Persahabatan Hospital, Jakarta, Indonesia, 2005-2008. Chest 138: 665-673.
16. Abdel-Ghafar AN, Chotpitayasunondh T, Gao Z, Hayden FG, Nguyen DH, et al. (2008) Update on avian influenza A (H5N1) virus infection in humans. N Engl J Med 358: 261-273.

17. Libraty DH, Acosta LP, Tallo V, Segubre-Mercado E, Bautista A, et al. (2009) A prospective nested case-control study of Dengue in infants: rethinking and refining the antibody-dependent enhancement dengue hemorrhagic fever model. PLoS Med 6: e1000171.
18. Sabchareon A, Wallace D, Sirivichayakul C, Limkittikul K, Chanthavanich P, et al. (2012) Protective efficacy of the recombinant, live-attenuated, CYD tetravalent dengue vaccine in Thai schoolchildren: a randomised, controlled phase 2b trial. Lancet 380: 1559-1567.
19. SEARO W (2011) Comprehensive Guidelines for Prevention and Control of Dengue and Dengue Haemorrhagic Fever. New Delhi: WHO SEARO.
20. Halstead SB (2012) Controversies in dengue pathogenesis. Paediatr Int Child Health 32 Suppl 1: 5-9.
21. Srikiatkhachorn A, Rothman AL, Gibbons RV, Sittisombut N, Malasit P, et al. (2011) Dengue--how best to classify it. Clin Infect Dis 53: 563-567.
22. Kalayanarooj S (2011) Dengue classification: current WHO vs. the newly suggested classification for better clinical application? J Med Assoc Thai 94 Suppl 3: S74-84.
23. Hadinegoro SR (2012) The revised WHO dengue case classification: does the system need to be modified? Paediatr Int Child Health 32 Suppl 1: 33-38.
24. Horstick O, Farrar J, Lum L, Martinez E, San Martin JL, et al. (2012) Reviewing the development, evidence base, and application of the revised dengue case classification. Pathog Glob Health 106: 94-101.

# Ringkasan

## Ringkasan

Setelah wabah sporadis terjadi pada abad ke-18 dan ke-19, *dengue* baru menjadi penyakit arbovirus terpenting setelah kasus DHF/DSS melanda Asia Tenggara selepas Perang Dunia II. Sejak saat itu, epidemiologi, virologi, imunologi, dan pathogenesis klinis, serta penatalaksanaan dan pencegahan infeksi virus *dengue* telah dipelajari oleh banyak peneliti di berbagai dunia. Namun banyak ketidaksesuaian pendapat serta gap (kepincangan) yang harus diselesaikan. Sebagai contoh, kalimat yang sering dikutip, "50-100 juta kasus *dengue* ditemukan di seluruh dunia setiap tahun", lebih tepat untuk menunjukkan seluruh infeksi dengue dimana juga termasuk infeksi dengue yang asimptomatik.

Berbagai kontroversi dan kepincangan pengetahuan lain masih ada, seperti kegunaan dan keterbatasan kriteria klinis WHO tahun 1997, 2009, dan 2011; diperlukan pengertian yang lebih baik mengenai patogenesis dari *dengue* yang berat (virulensi dari subtipe virus *dengue*, respons berlebihan atau tidak normal dari sel T, fenomena autoimun, serta infeksi sekunder); tidak tersedianya alat diagnostik yang cepat dan akurat, hewan uji coba, obat untuk virus, serta vaksin yang aman dan efektif.

Untuk mendapatkan pengertian yang lebih baik mengenai epidemiologi dan patogenesis dari infeksi *dengue*, diperlukan penelitian-penelitian prospektif dimana infeksi dengue termasuk kasus dengue yang berat dapat dengan tepat diidentifikasi dan dikarakterisasi. Berbagai jenis penelitian prospektif bisa dilakukan, di antaranya penelitian *kohort* berbasis populasi atau penelitian pada berbagai kelompok di lingkungan rumah dan penelitian berbasis rumah sakit. Penelitian *kohort dengue* yang berbasis populasi pertama kali dilakukan terhadap anak sekolah di Rayong, Thailand, pada 1984, diikuti dengan penelitian yang sama di Bangkok, Yangon di Myanmar, Yogyakarta di Indonesia, dan Managua di Nikaragua. Beberapa hasil yang didapatkan dari penelitian *kohort* ini antara lain pengertian yang lebih baik tentang beban penyakit *dengue* pada berbagai populasi, termasuk impak ekonomi dan karakterisasi determinan-determinan beratnya penyakit. Penelitian ini juga mengidentifikasi populasi dengan sejarah infeksi *dengue* yang diketahui

dengan baik, sehingga dapat menyediakan infrastruktur untuk pelaksanaan penelitian vaksin *dengue* fase ketiga. Kebutuhan untuk penelitian *kohort* pada orang dewasa juga diperlukan karena jumlah infeksi *dengue* yang ringan dan berat pada populasi ini semakin meningkat dalam tiga dekade terakhir, termasuk saat mewabah. Di samping itu, penelitian prospektif terhadap sekelompok orang di lingkungan rumah dilakukan untuk melihat transmisi di antara anggota keluarga dan dapat mendeteksi dengan cepat suatu infeksi *dengue* baru, sehingga dimungkinkan untuk mendapatkan *predictor* klinis dan imunologis dari penyakit yang berat.

Tesis ini menjelaskan soal hasil beberapa penelitian prospektif yang dilaksanakan di Jawa Barat, Indonesia. Yang pertama adalah suatu penelitian prospektif pada suatu *kohort,* yang terdiri atas 4.380 orang yang bekerja di tiga pabrik tekstil di Bandung, sepanjang 2000-2004 dan 2006-2009. Para pekerja ini dimonitor setiap hari dan diambil darahnya setiap 3-4 bulan sekali. Penelitian prospektif kedua dilaksanakan terhadap sekelompok orang di lingkungan rumah, yang terdiri atas anggota keluarga dan tetangga terdekat, yang berlangsung selama dua minggu, di mana gejala penyakit dari yang diduga terinfeksi *dengue* serta spesimen sebelum sakit dan selama observasi dikumpulkan. Penelitian ini dilaksanakan di Jakarta Barat pada 2001-2003. Tesis ini juga menyajikan temuan dari wabah *dengue* besar yang terjadi pada 2004, hasil dari *surveillance* influenza berskala nasional mulai 2003 sampai 2007 dan *surveillance* hantavirus di rumah sakit antara 2004 dan 2005. Semua penelitian ini didanai oleh United States Naval Medical Research Unit No. 2 (US-NAMRU#2), bekerja sama dengan Rumah Sakit Sumber Waras, Jakarta; Rumah Sakit Hasan Sadikin dan Fakultas Kedokteran Universitas Padjadjaran di Bandung; serta Badan Penelitian dan Pengembangan Kesehatan Kementerian Kesehatan di Jakarta.

**Bab 2** menyajikan aspek epidemiologi, virologi, dan klinis dari infeksi *dengue* pada suatu *kohort,* yang terdiri atas 4.380 pekerja dewasa di Bandung, Jawa Barat, yang diobservasi selama 79 bulan. Penelitian ini menunjukkan bahwa *dengue* menyumbang 12,41% dari seluruh kasus demam dan angka kejadian *dengue* sebesar 17,3 kasus/1.000 populasi setiap tahun. Angka ini 43 kali lebih besar daripada laporan nasional. Lebih jauh lagi, penelitian ini juga menunjukkan,

untuk angka infeksi *dengue* yang sebenarnya, perlu diperhitungkan angka kasus asimptomatik yang besarnya 2,6 kali dari angka kejadian *dengue* di atas. Data epidemiologis baru ini penting untuk diketahui oleh klinisi dan para pembuat kebijakan kesehatan, karena penelitian ini menunjukkan bahwa sangatlah penting memasukkan *dengue* ke diagnosis banding penyakit yang disertai demam, serta membuat strategi untuk mengendalikan penularan *dengue* yang ternyata lebih tinggi daripada yang sebelumnya diketahui. Di samping itu, *dengue* ditemukan sepanjang tahun, dengan puncaknya saat musim hujan, yaitu pada Desember-Maret. Semua serotipe virus *dengue* ditemukan setiap tahun, tapi keempat serotipe virus jarang ditemukan bersama-sama dalam satu bulan. Temuan ini memperlihatkan bahwa Bandung merupakan daerah yang *hyperendemic* untuk infeksi *dengue*. Dari 268 kasus yang terkonfirmasi, hanya satu kasus DSS yang didiagnosis. Sisanya adalah DHF derajat 1 dan derajat 2 serta bentuk yang ringan, yaitu demam *dengue* yang dapat atau tidak disertai manifestasi perdarahan. Kasus yang berat dalam penelitian ini berhubungan dengan DENV-3, yang secara konsisten telah dilaporkan sebagai penyebab utama wabah *dengue* di Indonesia. Data kami juga menunjukkan adanya keterkaitan antara kasus asimptomatik dan DENV-4. Dalam penelitian ini ditemukan pula terjadinya infeksi *dengue* berulang pada 7 subyek. Temuan ini menguatkan pendapat bahwa infeksi oleh serotipe tertentu tidak dapat melindungi dari infeksi dengue oleh serotipe lainnya. Akhirnya, penelitian ini tidak hanya menghasilkan data yang menunjukkan pentingnya *dengue* di Jawa Barat, tapi juga menjelaskan soal karakterisasi kejadian *dengue* secara alamiah pada populasi orang dewasa yang penting diketahui untuk penelitian vaksin di masa yang akan datang.

Sementara **Bab 2** menjabarkan data infeksi *dengue* yang terjadi secara alamiah pada keadaan endemik, **Bab 3** memaparkan ihwal data saat terjadi wabah *dengue* pada 2004, di mana dilaporkan sebanyak 50.000 kasus dengan 603 kematian. Data tersebut menjadi penting karena wabah *dengue* terjadi secara periodik sejak 1968. Bab ini menyajikan data epidemiologi, virologi, dan klinis dari 10 rumah sakit di lima wilayah di Jakarta, Indonesia. Penelitian ini memastikan bahwa persebaran wabah terjadi di semua wilayah, dan keempat serotipe ditemukan bersama-sama saat wabah ini muncul dengan DENV-3 yang paling dominan. Analisis genotipe menunjukkan bahwa DENV-3 yang beredar identik dengan yang menyebabkan wabah pada 1998. Beredarnya seluruh serotipe dengan

DENV-3 sebagai yang paling dominan telah dilaporkan saat muncul wabah-wabah sebelumnya, termasuk saat wabah terbesar pada 1998. Keadaan ini, di samping adanya populasi yang sama sekali belum pernah terinfeksi *dengue* dan bersirkulasinya *strain* virus baru atau yang lebih virulen, mungkin merupakan kondisi yang diperlukan untuk terjadinya wabah *dengue* di suatu daerah endemis. Ditemukan pula bahwa wabah mengenai seluruh golongan umur dan proporsi DHF jauh lebih tinggi saat terjadi wabah dibanding kondisi endemik. Padahal kemungkinan tidak terdiagnosisnya kasus DHF cukup besar karena kurangnya data hematologis serial untuk menunjukkan adanya hemokonsentrasi. Hal lain yang ditemukan adalah besarnya jumlah kasus *dengue* yang tidak bisa dipastikan, sama seperti laporan sebelumnya dari Semarang.

Pada dua bab pertama telah dijabarkan bahwa Jawa Barat merupakan suatu wilayah *hyperendemic,* di mana keempat serotipe virus *dengue* bisa ditemukan. Merujuk pada teori *antibody dependent enhancement theory (ADE),* keadaan ini menyebabkan populasi di sini mempunyai risiko yang lebih tinggi timbulnya penyakit yang lebih berat saat terkena infeksi *dengue* kedua kalinya. Pada **Bab 4**, bukti-bukti terjadinya infeksi *dengue* tiga kali berturut-turut oleh serotipe yang berbeda-beda pada empat subyek telah dijabarkan. Infeksi pertama adalah sebelum masuk ke studi dan dua infeksi berikutnya saat berada dalam studi. Subyek ini berumur 29-42 tahun dan, sebelum terinfeksi kedua kalinya, mereka tidak atau hanya mempunyai antibodi netralisasi yang rendah terhadap serotipe virus yang menginfeksi tersebut. Hanya satu di antara keempat kasus berakhir dengan infeksi sekunder yang berat (DHF Grade II). Dalam kasus ini, sebelum sakit untuk kedua kalinya, subyek menunjukkan bukti pernah terkena infeksi virus DENV-2 dan adanya titer antibodi yang rendah terhadap virus yang kemudian menginfeksinya, DENV-4 ($PRNT_{50}$ 29). Urutan serotipe yang menginfeksi pada ketiga subyek lainnya ketika mereka hanya mengalami demam *dengue* adalah: DENV-1 ke DENV-3, DENV-2 ke DENV-3, dan DENV-2 ke DENV-4. Untuk infeksi ketiga, semua hanya menyebabkan infeksi *dengue* yang ringan. Pada dua kasus DENV-3 terjadi setelah infeksi oleh DENV-4. Sementara untuk dua kasus, serotipe ketiga tidak dapat diketahui. Serum yang diambil sebelum infeksi ketiga menunjukkan adanya antibodi netralisasi terhadap seluruh serotipe virus. Waktu antara infeksi kedua dan ketiga pada keempat subyek ini adalah 5, 11, 18, dan 18 bulan. Bentuk yang lebih ringan pada infeksi ketiga ini mungkin ada

hubungannya dengan durasi viremia yang lebih pendek karena hanya satu dari empat kasus virus *dengue* bisa dideteksi. Penelitian ini memberi bukti bahwa adanya antibodi netralisasi yang *cross-reactive* tidak dapat melindungi dari infeksi *dengue* pada masa yang akan datang. Sebaliknya, seperti yang ditemukan pada satu kasus, bisa timbul kasus yang lebih berat (DHF).

Mengingat infeksi *dengue* primer merupakan faktor risiko terjadinya infeksi sekunder dengan gejala klinis yang berat, penting membedakan kedua jenis infeksi ini. Berdasarkan petunjuk dari WHO, uji *hemagglutination inhibition* (HI) bisa digunakan. Pada **Bab 5**, kemampuan uji HI untuk membedakan infeksi primer dan sekunder dibandingkan dengan uji antibodi IgG ELISA menggunakan *plaque reduction neutralization antibody test* (PRNT) sebagai standar emas. Uji IgG ELISA direkomendasikan karena hasilnya sesuai dengan PRNT dan lebih mudah dilakukan. Penelitian ini menunjukkan bahwa uji HI masih bisa digunakan, tapi kriterianya perlu direvisi. Berdasarkan hasil penelitian ini, titer di bawah ≤80 pada serum konvalesen menunjukkan infeksi primer, sedangkan titer >640 adalah infeksi sekunder. Titer di antara 160 dan 640 pada serum konvalesen sulit diinterpretasikan sehingga diperlukan uji lanjutan dengan IgG ELISA atau PRNT. Di samping untuk menentukan infeksi primer atau sekunder, uji HI juga digunakan untuk mengkonfirmasi infeksi *dengue* itu sendiri. Namun uji ini tidak praktis dalam pelaksanaannya, lantaran dapat menunjukkan hasil yang *cross reactive* dengan infeksi Flavivirus lain, seperti *Japanese encephalitis*, dan hanya memberikan diagnosis retrospektif.

Dengan demikian, diperlukan sebuah uji diagnostik yang lebih baik. Standar emas yang ada, seperti kultur virus dan uji isolasi virus, secara teknis sulit dilakukan dan membutuhkan waktu yang lama. Sementara itu, uji molekuler, seperti RT-PCR, bisa diandalkan, tapi biayanya mahal dan hanya dapat dilakukan di laboratorium besar atau institusi penelitian. Akhirnya uji serologis, khususnya uji diagnostik cepat, sangat mudah dilaksanakan, tapi hasil antibodi IgM hanya bisa diinterpretasikan jika spesimen diambil lima hari sesudah demam muncul. Pada kasus infeksi sekunder, uji IgG menggunakan spesimen akut dan konvalesen diperlukan karena sering kali antibodi IgM tidak dapat dideteksi. Untuk itu, beberapa uji komersial untuk mendeteksi protein *dengue non-structural* 1 (NS1) telah dibuat. Protein NS1 disandi oleh genom virus dan kemudian disekresikan

oleh sel yang terinfeksi dalam bentuk terlarut. NS1 ini dapat dideteksi pada fase awal penyakit, yaitu beberapa hari sebelum munculnya antibodi IgM. Uji ini relatif murah, mudah dikerjakan, dan cepat selesai. Pada **Bab 6**, nilai diagnostik dan prediktif dari uji antigen NS1 untuk diagnosis dini infeksi *dengue* dievaluasi. Penelitian ini menemukan bahwa sensitivitasnya rendah, yaitu 46,8% atau terendah dibanding hasil penelitian dari negara lain. Namun, karena sensitivitas untuk infeksi primernya sangat baik, yaitu 100%, uji ini sangat berguna dalam kedokteran wisata. Sensitivitas uji NS1 juga bervariasi antar-serotipe yang menginfeksi, tertinggi pada DENV-3 (47,1%), diikuti DENV-1 (40,9%), DENV-2 (30%), dan DENV-4 (27%). Lebih jauh lagi, penelitian ini menemukan bahwa antigen NS1 lebih jarang terdeteksi pada mereka yang telah terkena infeksi *dengue* oleh dua atau lebih serotipe. Sedangkan antigen NS1 lebih sering dideteksi pada wanita, dan pada kasus yang berat (*dengue fever* dengan manifestasi perdarahan dan DHF), serta subyek dengan jumlah trombosit yang rendah (<100.000/mm3).

Adanya suatu alat diagnostik yang cepat, akurat, murah, serta dapat mendeteksi dini infeksi *dengue*, selain berguna untuk penatalaksanaan pada pasien dan komunitas, bisa membantu untuk membuat diagnosis yang lain jika *dengue* tidak dapat ditegakkan. **Bab 7, 8, 9,** dan **10** menjabarkan empat penyakit infeksi yang perlu dipertimbangkan oleh para klinisi di Jawa Barat sebagai diagnosis banding pada pasien dengan demam di mana gejala dan tanda penyakitnya sulit dibedakan antara satu dan yang lain. **Bab 7** melaporkan infeksi chikungunya yang ditemukan di antara subyek penelitian orang dewasa di Bandung. Tidak seperti penelitian sebelumnya, bahwa chikungunya sering dilaporkan dari kejadian wabah di suatu tempat, penelitian kami menemukan infeksi chikungunya yang terus-menerus, yang menunjukkan bahwa virus ini endemik di Bandung. Karena proporsinya pada penderita demam cukup tinggi (7,1%), chikungunya perlu tetap dipikirkan sebagai salah satu diferensial diagnosis, meskipun tidak ditemukan *arthralgia*. Kami menemukan pula kemungkinan adanya infeksi chikungunya berulang pada tiga subyek, yang menunjukkan bahwa antibodi chikungunya dari infeksi sebelumnya mungkin tidak dapat memberi kekebalan seumur hidup dan/atau adanya *strain* berbeda yang beredar di Bandung. Temuan penting lainnya adalah antibodi IgM yang bertahan untuk waktu yang lama mempersulit diagnosis dengan serologis karena dibutuhkan spesimen akut dan konvalesen untuk melihat kenaikan titer antibodi.

**Bab 8** membahas influenza, termasuk influenza A/H5N1 yang sangat patogenik, sebagai salah satu diagnosis diferensial dari *dengue*. Data didapatkan dari program *surveillance* nasional sejak 2003 sampai 2007. Pada *surveillance* nasional ini terdapat enam tempat penelitian yang terletak di tiga kota di Jawa Barat. Influenza dikonfirmasi pada 20,1% dari subyek yang datang dengan *influenza like illness*. Tidak seperti di negara dengan empat musim, influenza ditemukan sepanjang tahun di Indonesia dengan puncaknya pada musim hujan antara Desember dan Maret. Kepentingan untuk dapat membedakan *dengue* dengan influenza bukan hanya karena penatalaksanaan untuk pasien yang berbeda, tapi juga bagaimana upaya pencegahan di keluarga, sekolah, dan tempat kerja yang berbeda. Hal ini khususnya sangat penting berkaitan dengan infeksi influenza A/H5N1, dengan angka kematian 82%, tapi hasilnya dapat lebih baik jika Oseltamivir diberikan lebih dini. Influenza A/H5N1 sering kali tidak dipikirkan di awal sakit. Malah sering didiagnosis sebagai penyakit lain (*dengue*, demam *typhoid*, *diarrhea*, infeksi saluran napas, dan lainnya). Pasien dengan badan panas dan sesak napas sering salah didiagnosis sebagai *dengue* dengan edema paru. *Surveillance* nasional ini menemukan pula adanya *antigenic drift* pada *strain* influenza A dan B, yang sama dengan *strain* vaksin yang digunakan oleh WHO, serta dideteksi lebih awal daripada *strain* vaksin tersebut.

**Bab 9** membahas soal penemuan infeksi *Seoul virus*, yang termasuk dalam kelompok hantavirus, berdasarkan data klinis, serologis, dan epizoologis. Gejala dan tanda penyakit yang ada, seperti demam, nyeri otot, perdarahan, *leucopenia*, *thrombositopenia*, serta peningkatan SGOT dan SGPT, konsisten dengan infeksi hantavirus. Namun, karena mirip dengan gejala dan tanda pada kasus *dengue* yang berat, kedua penyakit ini menjadi sulit dibedakan. Dengan demikian, infeksi hantavirus ini didiagnosis dengan adanya peningkatan antibodi IgM dan IgG dan, pada saat yang sama, tidak ditemukan bukti adanya infeksi *dengue*. Di samping itu, diagnosis hantavirus diperkuat oleh tingginya prevalensi rodensia, yang mempunyai antibodi IgG terhadap hantavirus di lingkungan tempat tinggal pasien tersebut, dibanding di daerah kontrol (13,2% vs 4,7%, p=0.04) serta terdeteksinya virus Seoul pada organ dari 71% rodensia yang memiliki antibodi IgG terhadap hantavirus.

**Bab 10** menyajikan bukti penemuan *West Nile virus* (WNV) pertama di Indonesia. Virus ini ditemukan dari spesimen arsip yang dikumpulkan saat penelitian penyakit hantavirus di dua rumah sakit di Bandung pada 2004-2005. Virus WNV yang positif berasal dari spesimen seorang anak laki-laki berumur 15 tahun dan masuk ke rumah sakit dengan panas badan, epistaksis, gangguan pencernaan, peningkatan SGOT dan SGPT, *leucopenia,* serta *thrombositopenia*. Tidak ditemukan gejala neurologis, dan pasien pulang dalam keadaan sembuh. Analisis genetik menunjukkan bahwa virus yang ditemukan ini mirip dengan *strain* WNV, yang pertama kali diisolasi dari Uganda dan termasuk dalam *lineage* 2. *Lineage* ini lebih rendah virulensinya ketimbang *lineage* 1, yang merupakan penyebab umum *encephalitis virus* di Amerika Serikat, dan biasanya berkaitan dengan wabah besar.

Di samping virus *dengue*, penyebab-penyebab infeksi lainnya yang dibahas pada **Bab 7-10** adalah hanya empat dari banyak virus lain yang perlu dipertimbangkan sebagai diagnosis diferensial pada pasien yang datang dengan demam di Indonesia. Dibuatnya suatu alat uji cepat yang dapat membedakan antara infeksi virus dan bakteri akan sangat bernilai karena dapat membantu dalam pemberian anti-mikroba yang lebih rasional dan penatalaksanaan pasien dengan demam. Lebih jauh lagi, karena *dengue* merupakan penyakit yang prevalens dan kadang-kadang disertai penyulit, suatu alat uji diagnostik yang akurat untuk memastikan infeksi *dengue* atau memastikan bukan infeksi *dengue* dalam kasus demam akut sangatlah dibutuhkan.

Pembuatan suatu vaksin *dengue* terhambat oleh keharusan memproteksi keempat serotipe tanpa membuat penerima vaksin berisiko mengalami ADE. Cara pencegahan di komunitas yang saat ini ada adalah pemberantasan sarang nyamuk, peningkatan pengetahuan untuk meningkatkan kewaspadaan, langkah awal yang perlu dilakukan, kapan melapor kepada petugas kesehatan, serta tanda-tanda penting sebagai indikasi untuk mengirim seorang pasien ke rumah sakit. Dua penelitian menyediakan informasi yang berharga sehubungan dengan langkah preventif di masyarakat. Penelitian pertama bertujuan meningkatkan pengetahuan dan kesadaran akan *dengue* pada populasi orang dewasa yang bekerja di pabrik tekstil di Bandung. Penelitian kedua adalah observasi klinis dan

virologi di masyarakat untuk mengantisipasi kasus lainnya setelah satu kasus indeks ditemukan.

**Bab 11** menunjukkan bagaimana memasukkan pendidikan di dalam penelitian *dengue* prospektif telah mempengaruhi pengetahuan peserta mengenai *dengue*. Pendidikan yang diberikan dilakukan dalam berbagai bentuk, seperti penyuluhan, poster, dan brosur, yang menjelaskan perihal gejala dan tanda-tanda *dengue*, bagaimana cara penularannya, serta bagaimana cara pencegahannya. Saat peserta masuk ke penelitian, kuesioner disampaikan secara lisan oleh dokter penelitian untuk melihat bagaimana pengetahuan peserta mengenai *dengue*. Kuesioner yang sama ditanyakan kembali 18 bulan kemudian. Dari 2.340 peserta yang menyelesaikan kedua tes, hanya 0,3% yang mendapatkan hasil "sangat baik" pada *pre-test,* sedangkan 8,4% mendapatkan nilai "sangat baik" pada *post-test*. Jumlah peserta yang mendapatkan nilai "sangat buruk" berkurang pada *post-test* dibanding pada *pre-test* (1,4% vs 4,0%). Nilai rata-rata saat *pre-test* dan *post-test* adalah 7,8 dan 10,1, dari kemungkinan maksimum. Pendidikan berkaitan erat dengan nilai yang didapatkan. Hasil ini menunjukkan bahwa penelitian prospektif ini dapat meningkatkan pengetahuan tentang *dengue* pada subyek penelitian.

**Bab 12** memaparkan hasil pengamatan selama dua minggu terhadap keluarga dan tetangga dekat dari 53 kasus yang terkena *dengue* di Jakarta Barat pada 2002-2003. Dari 785 anggota keluarga atau tetangga yang diamati, 192 (24,5%) infeksi *dengue* terpantau, terdiri atas 175 kasus (22,3%) yang telah terjadi sebelum pengamatan dimulai dan 17 kasus (2,2%) muncul pada saat pengamatan. Dari 17 kasus baru tersebut, 8 adalah infeksi asimptomatik, 8 demam *dengue,* dan 1 DHF *grade* II. Penelitian ini memperlihatkan bahwa satu orang yang dirawat di rumah sakit berkaitan dengan 3,6 kasus *dengue* yang terdapat di lingkungan dekat tempat tinggal kasus tersebut. Hal ini menunjukkan pentingnya keikutsertaan masyarakat untuk melaksanakan langkah-langkah pencegahan dan pengamatan. Di samping itu, penelitian ini juga menunjukkan pentingnya kasus asimptomatik dalam menyebarkan penularan virus *dengue* karena viremia ditemukan pada dua dari delapan kasus asimptomatik.

# Samenvatting

## Samenvatting

Tijdens de 18e en de 19e eeuw kwam knokkelkoorts meestal sporadisch voor terwijl na de Tweede Wereldoorlog ernstige DHF/ DSS uitbraken werden gerapporteerd in Zuidoost Azië waarna Dengue algemeen erkend werd als de belangrijkste Arbovirus infectie. Sindsdien is de epidemiologie, virologie, immunologie, pathogenese, preventie en behandeling van knokkelkoorts door verscheidende onderzoekers op verschillende continenten onderzocht en bestudeerd. Ondanks dit onderzoek, blijven er vele aspecten van knokkelkoorts onopgehelderd. Zo wordt vaak gerapporteerd dat "elk jaar wereldwijd ongeveer 50 -100 miljoen gevallen van knokkelkoorts ontstaan ", terwijl dit mogelijk niet overeen komt met de werkelijkheid omdat een groot aantal infecties asymptomatisch verloopt.

Behalve onduidelijkheden in de epidemiologie, zijn er ook veel tegenstrijdigheden kennis lacunes over andere aspecten van de knokkelkoorts, zoals het nut en de beperkingen van de WHO-criteria voor klinische classificaties van de jaren 1997, 2009 en 2011; het inzicht in the pathogenese van ernstige dengue (virulentie van dengue virus subtypen, abnormale of versnelde T cel reacties, auto-immune verschijnselen of tweede infecties); de onbeschikbaarheid van snelle en nauwkeurige diagnostische tests; dier modellen; antivirale middelen; en de beschikbaarheid van een veilige en doeltreffend vaccin.

Om ons inzicht wat betreft de epidemiologie en pathogenese van knokkelkoorts te verbeteren, zijn prospectieve studies nodig, die nauwkeurig de ernst van knokkelkoorts infecties kunnen identificeren en typeren. Verschillende soorten prospectieve studies kunnen daarvoor gebruikt worden, zoals grootschalige cohortstudies of clusterstudies in gemeenschappen evenals ziekenhuisgerelateerde studies. De eerste grootschalige cohortstudie werd in 1984 uitgevoerd onder schoolkinderen in Rayong - Thailand, gevolgd door vijf vergelijkbare studies uitgevoerd in Bangkok en andere steden, waaronder Yangon in Myanmar, Yogyakarta in Indonesië en Managua in Nicaragua.

Deze cohortstudies leverden allerlei resultaten op, zoals een beter inzicht over

de ziektelast in de verschillende bevolkingsgroepen, de economische effecten en inzicht in de determinanten die de ernst van de ziekte bepalen. Bovendien werden populaties bestudeerd waarvan bekend was dat knokkelkoorts reeds langere tijd aanwezig was: gedegen kennis van deze populaties kunnen nuttig zijn voor het uitvoeren van fase III vaccin studies.

De noodzaak om longitudinale cohortstudies bij volwassenen uit te voeren werd ook duidelijk toen de laatste drie decennia het aantal ongecompliceerde en gecompliceerde knokkelkoorts infecties bij volwassenen toenam. Als gevolg daarvan werden binnen bepaalde gemeenschappen prospectieve clusterstudies uitgevoerd om besmetting onder leden van het huishouden te kunnen bestuderen en om infecties in de beginfase te kunnen traceren waardoor de klinische en immunologische voorspellers van een ernstige ziekte geïdentificeerd konden worden.

Dit proefschrift geeft een beschrijving van de resultaten van verschillende prospectieve studies, zoals uitgevoerd in West-Java, Indonesië.

De eerste studie betreft een prospectieve cohortstudie. Deze is in de periodes 2000—2004 en 2006-2009 uitgevoerd in drie textielfabrieken te Bandung, waarbij 4380 volwassenen betrokken waren, die dagelijks werden gecontroleerd en waarbij elk kwartaal bloed afgenomen werd.

De tweede studie is een prospectieve clusterstudie in een gemeenschap in West-Jakarta, uitgevoerd tijdens de periode 2001-2003, waarbij familieleden en buren van een dengue geïndiceerde patiënt gedurende twee weken werden gevolgd met als doel klinische informatie te verzamelen zowel als bloed monsters.

Dit proefschrift geeft bovendien ook informatie over een grote knokkelkoorts uitbraak in 2004, de nationale influenza surveillance 2003-2007, en een ziekenhuisgerelateerde hantavirus surveillancestudie welke uitgevoerd is tussen 2004 en 2005. Deze studies werden gefinancierd door Naval Medical Research Unit 2 uit de Verenigde Staten (U.S.-NAMRU #2) in samenwerking met het Sumber Waras ziekenhuis in Jakarta, het Hasan Sadikin Hospital en Faculty of Medicine

Universitas Padjadjaran in Bandung, en het National Institute of Health Research and Development van het ministerie van Volksgezondheid van Indonesië in Jakarta.

**Hoofdstuk 2** beschrijft de epidemiologie, virologie en klinische aspecten van dengue infecties, zoals die werden geobserveerd gedurende een periode van 79 maanden in een cohort van 4380 volwassen fabrieksarbeiders uit Bandung te West-Java. Deze studie toont aan dat knokkelkoorts in 12.41% de oorzaak is van de koorts terwijl de jaarlijkse incidentie van dengue 17.3 gevallen/1000 inwoners was. Deze studie toont verder aan- dat bij de berekening van het werkelijke aantal dengue-infecties per inwoners per jaar - ook berekend moet worden de asymptomatische infecties, die 2.6x groter was dan de symptomatische geïndiceerde gevallen.

Deze nieuwe epidemiologische gegevens zijn belangrijk voor clinici en gezondheidsmedewerkers, omdat zij aantonen dat het belangrijk is om knokkelkoorts op te nemen in de differentiële diagnose van ongedifferentieerde koorts. Verder werden gedurende het gehele jaar knokkelkoorts gevallen gevonden met een piek tijdens het regenseizoen dat meestal van december tot maart plaats vindt. Alle serotypes werden gedurende het gehele jaar aangetoond, hoewel ze zelden gelijktijdig in dezelfde maand aanwezig waren.

Tevens werd aangetoond dat Bandung een hyper-endemische Dengue gebied is. Uit een totaal van 268 bevestigde gevallen werd slechts één DSS geval gediagnosticeerd. De overige waren DHF fase I en II en de milde vorm van dengue koorts met of zonder hemorragische manifestaties. Ernstige gevallen werden geassocieerd met DENV-3, het serotye dat als de belangrijkste oorzaak van uitbraken in Indonesië wordt gerapporteerd. In deze studie vonden we ook herhaalde knokkelkoorts infecties bij zeven patiënten. Deze bevinding bevestigt dat een infecties met een bepaalde serotype, het individu niet tegen infecties met andere serotypes beschermd. Tot slot heeft onze studie niet alleen waardevolle gegevens opgeleverd voor het belang van knokkelkoorts in West-Java, maar heeft ook het natuurlijke beloop van de ziekte gekarakteriseerd bij volwassenen, wat belangrijk is voor toekomstige vaccin studies.

Terwijl **hoofdstuk 2** gegevens vermeld over de endemische aard van knokkelkoorts infecties, wordt in **Hoofdstuk 3** een denque uitbraak uit 2004 in Indonesië beschreven, waarbij 50.000 gevallen en 603 doden werden gerapporteerd. Het hoofdstuk bevat epidemiologische, klinische en virologische gegevens uit 10 ziekenhuizen uit vijf deelgemeenten van Jakarta. Onze bevindingen bevestigen dat alle deelgemeenten werden getroffen door deze uitbraak, terwijl alle vier de knokkelkoorts serotypes tegelijkertijd - met DENV-3 als de meest overheersende- in omloop waren. Genotype analyse gaf aan dat de circulerende DENV-3 identiek was aan het type dat in 1998 de epidemie had veroorzaakt. Gelijktijdige circulatie van meerdere serotypes -met DENV-3 als de meest overheersende- werd ook beschreven in vorige knokkelkoorts uitbraken in Indonesië, zoals gedurende de grootste uitbraak in 1998.

Ons onderzoek toont ook aan dat alle leeftijdsgroepen getroffen werden en dat het aandeel van DHF significant hoger was tijdens een uitbraak vergeleken met "niet-uitbraak" omstandigheden, ondanks de mogelijke onderschatting van DHF gevallen vanwege het ontbreken van serieel bloedonderzoek om hemoconcentratie aan te tonen. Ten slotte, net als bij een eerder rapport uit Semarang, kon de diagnose knokkelkoorts in een groot deel (33%) van de gevallen niet bevestigd worden.

In de eerste twee hoofdstukken wordt West- Java beschreven als een hyper-endemische regio waar alle vier de DENV serotypes circuleerden. Volgens de antibody afhankelijkheid versterkingstheorie (ADE) is het risico voor het ontwikkelen van ernstige ziekte onder dergelijke omstandigheden verhoogd. In **hoofdstuk 4** worden drie heterologe opeenvolgende knokkelkoorts infecties bij vier personen beschreven. De eerste infectie was voorafgaand aan hun deelname aan de studie en de tweede infectie was na inclusie in de studie. Deze patiënten met een leeftijden van 29-42 jaar hadden voorafgaand aan hun secondaire infecties geen (of slechts een lage) titer van neutraliserende antibodies tegen het serotype. Slechts één van de vier secundaire infecties resulteerde in een ernstigere DHF graad II.

Voorafgaand aan de secundaire infectie had deze patiënt een aangetoonde

doorgemaakte DENV-2 infectie en een lage titer ($PRNT_{50}$ 29) voor het serotype (DENV-4). De volgorde van de serotypes bij de andere studiedeelnemers - die alleen dengue koorts ontwikkelden - varieerde van DENV-1 tot DENV-3, DENV-2 tot DENV-3 en DENV-2 tot DENV-4. Alle tertiaire infecties resulteerden in een milde vorm van dengue infectie. In twee gevallen volgde DENV-3 na een vorige DENV-4 infectie, terwijl in de andere twee gevallen, de serotypes van de derde infectie niet kon worden vastgesteld. Bloedmonsters voorafgaand aan de ziekte vóór de derde infecties toonde neutraliserende antilichamen tegen alle serotypes. De intervallen tussen de tweede en derde infecties waren respectievelijk 5, 11, 18 en 18 maanden.

Een mildere vorm van ziekte tijdens tertiaire infecties kan mogelijk correleren met de korte duur van de viremie, omdat het knokkelkoortsvirus alleen in één van de vier gevallen geïsoleerd kon worden. Al met al levert onze studie het bewijs dat de aanwezigheid van kruisreagerende neutraliserende antilichamen niet perse tegen toekomstige knokkelkoorts infecties beschermt. Integendeel, zoals aangetoond in één geval, een ernstiger beloop kan daarbij ook optreden (DHF).

Omdat een primaire dengue infectie één van de risicofactoren is voor het ontwikkelen van ernstige secundaire infecties, is het belangrijk onderscheid te maken tussen deze twee soorten infecties. Volgens de richtlijnen van de WHO kan de hemagglutinatie remmingstest (HI) hiervoor worden toegepast.

In **hoofdstuk 5** wordt onderzocht of de HI test het onderscheid kan maken tussen een primaire en secundaire infectie. De HI test werd daarbij vergeleken met de IgG ELISA en de Plaque Reduction Neutralization antibody Test (PRNT) werd als Gouden Standaard gebruikt. De ELISA IgG test wordt vaak aanbevolen omdat de resultaten in vergelijking met PRNT eenvoudiger uit te voeren zijn. We vonden dat de HI-test met behulp van gemodificeerde criteria gebruikt kon worden waarbij titers ≤80 overeenkomen met een primaire infectie terwijl titers > 640 als secundaire infecties moeten worden beschouwd. Titers tussen 160 en 640 zijn moeilijker te interpreteren. Andere testen- zoals de IgG ELISA of PRNT - kunnen in deze gevallen gebruikt worden.

Afgezien van bovengenoemde indicatie, wordt de HI-test ook aanbevolen voor de bevestiging van de knokkelkoorts diagnose. Probleem is echter dat de test tamelijk omslachtig is en er kruisreacties kunnen zijn met andere Flavivirussen zoals Japanse encephalitis. Vaak levert deze test dus alleen een retrospectieve diagnose op.

Al met al is er dus een grote behoefte aan betere diagnostische testen om de diagnose knokkelkoorts in het veld te kunnen stellen. De gouden standaard viruskweek en isolatie analyse zijn technisch gezien moeilijk en tijdrovend. Bovendien zijn robuuste moleculaire tests zoals RT-PCR duur en kunnen ze alleen uitgevoerd worden in grote laboratoria of onderzoeksinstituten. Ten slotte zijn serologische tests, met name de snelle diagnostische tests, gemakkelijk uit te voeren. Echter, de specifieke Dengue IgM antilichamen zijn pas aantoonbaar vijf dagen na de eerste ziekte verschijnselen.

In secundaire gevallen zijn gepaarde IgG testen vaak vereist als IgM antilichamen niet kunnen worden aangetoond. Recentelijk zijn verschillende commerciële testen ontwikkeld die zich richten op het aantonen van het niet-structurele 1 (NS1) proteïne van het knokkelkoortsvirus. NS1 wordt gecodeerd door het virusgenoom vervolgens uitgescheiden door geïnfecteerde cellen in een oplosbare vorm. NS1 is aantoonbaar in het bloed gedurende eerste dagen van de ziekte, voordat anti-dengue IgM-antistoffen waarneembaar zijn. De test is relatief goedkoop, gemakkelijk uit te voeren en snel. In **hoofdstuk 6** wordt de diagnostische en voorspellende waarde van de NS1 antigeentest bij vroege diagnose van een dengue infectie besproken. We vonden een sensitiviteit van 46.8%, wat de laagste was vergeleken studies uit andere landen. Desalniettemin was de sensitiviteit bij een primaire infectie 100%. De sensitiviteit varieerde tevens onder de verschillende serotypes, waarbij de hoogste sensitiviteit werd gevonden bij DENV-3 (47.1%), gevold door DENV1- (40.9%), DENV-2 (30%) en DENV-4 (27%). Verder vonden we dat NS1 minder frequent gedetecteerd werd bij patiënten met eerdere infecties met meer dan één serotype, terwijl NS1 vaker voorkwam bij vrouwen, ernstigere gevallen (denguekoorts met hemorragische verschijnselen en DHF) en patiënten met weinig trombocyten (<100.000/mm3).

In de **Hoofdstukken 7**, **8**, **9** en **10** bespreken we vier besmettelijke ziekten die onderdeel uitmaken van de differentiële diagnose van ongedifferentieerde koorts in West-Java. **Hoofdstuk 7** gaat over chikungunya virus infecties bij volwassenen in Bandung. In tegenstelling tot eerdere studies waar gemeld wordt dat chikungunya als focale epidemie voorkomt, vond onze studie doorlopend chikungunya infecties. Bovendien bleek dat het aandeel van chikungunya infecties onder gevallen van acute febriele aandoening hoog is (7,1%), terwijl gewrichtspijn niet als een een prominent symptoom gerapporteerd wordt. We hebben ook het herhaalde malen voorkomen van chikungunya infecties bij drie patiënten aangetoond dat mogelijk is omdat ofwel chikungunya antibodies van vorige infecties niet levenslang immuniteit kunnen bieden kunnen en/of er mogelijk verschillende stammen in Bandung circuleren. Tot slotte bleek uit ons onderzoek dat IgM-antistoffen langdurig kunnen voorkomen hetgeen het stellen van de diagnose chikungunya compliceert.

**Hoofdstuk 8** gaat over influenza, inclusief de hoog pathogene influenza A/H5N1. Data werden verzameld uit een nationale influenza onderzoek - uitgevoerd van 2003 tot 2007- waaronder gegevens van zes locaties van drie steden in West-Java. Bij patiënten met influenza-achtige ziekte verschijnselen, kon bij 20,1% van de patiënten influenza worden aangetoond. In tegenstelling tot landen met een gematigd klimaat, circuleren in Indonesië gedurende het hele jaar influenzavirussen maar er is een piek in het regenseizoen van December tot Maart. Het is belangrijk om dengue en influenza van elkaar te onderscheiden: niet alleen omdat de behandeling anders is, maar ook omdat voorzorgsmaatregelen voor familie, school of werkplaats verschillend zijn. Dit onderscheid is vooral belangrijk met betrekking tot influenza A/H5N1, dat een sterftecijfer kan hebben dat kan oplopen tot 82%, maar waarvan de uitkomst gunstiger is wanneer Oseltamivir eerder word gegeven. Influenza A/H5N1 werd vaak niet overwogen bij patiënten met acute koorts omdat alternatieve diagnoses werden gemaakt (knokkelkoorts, tyfus, diarree ziekte, infectie van de luchtwegen). Aan de andere kant werd bij koortsende patiënten met kortademigheid, knokkelkoorts met longoedeem vaak ten onrechte gediagnosticeerd. Tijdens deze nationale surveillance studies bleek ook dat de antigenen van de circulerende virusstammen van influenza A en B veranderden.

**Hoofdstuk 9** worden middels klinische, serologische en epizoölogische gegevens, de eerste Indonesische Seoul virusinfectie - die tot het geslacht hantavirus behoort, gedocumenteerd. Tekenen en symptomen, hoewel overeenkomend met hantavirus infectie - zoals koorts, spierpijn, bloedingsneiging, leukopenie, trombocytopenie, toegenomen serum transaminase- lijken vaak op ernstige een dengue infectie. Deze twee infectieziekten zijn daarom moeilijk te onderscheiden. Hantavirus infectie werd gediagnosticeerd door aantonen verhoogde hantavirus IgM en IgG antistoffen, terwijl de diagnose dengue-infectie verworpen kon worden. De diagnose van hantavirus infecties werd bovendien ook ondersteund doordat de prevalentie van hantavirus IgG antilichamen bij knaagdieren gevangen in de nabijheid van de patiënt zijn huis, hoger bleek te zijn dan bij knaagdieren uit een controlegebied (13,2% vs. 4,7%, $p = 0.04$). Bovendien werd het Seoul virus in 71 % van de seropositieve knaagdieren aangetoond.

In **hoofdstuk 10** wordt voor de eerste keer het voorkomen van het West-Nijl virus (WNV) in Indonesië aangetoond. Het virus werd ontdekt in de gearchiveerde monsters van een studie uit 2004-2005 naar de oorzaken van acute koortsende ziekte bij gehospitaliseerde patiënten in Bandung, West-Java Indonesië. Het WNV positieve monster was afkomstig van een 15-jarige jongen opgenomen met epistaxis, gastro-intestinale symptomen, verhoogde serum transaminase, leukopenie en trombocytopenie. Er werden geen neurologische symptomen gemeld en de patiënt is volledig hersteld zonder restverschijnselen.

Behalve knokkelkoorts virus en de andere virussen beschreven in de **hoofdstukken 7** tot en met 10, moeten er nog vele andere micro-organismen meegenomen worden in de differentiële diagnose bij patiënten met koortsende ziektes in Indonesië. De ontwikkeling van "point of care testen" - die bacteriële van virale infecties van elkaar onderscheiden, kunnen daarom van groot belang zijn voor het rationeel voorschrijven van antimicrobiële geneesmiddelen en de zorg voor dergelijke patiënten. Aangezien knokkelkoorts een veel voorkomend ziekte is - met soms een gecompliceerde verloop- is een robuuste diagnostische point of care test voor het aantonen of het uitsluiten van denque dringend nodig. De ontwikkeling van een dengue vaccin wordt gecompliceerd doordat het vaccin bescherming moet bieden aan alle vier de serotypen zonder de gevaccineerde

personen bloot te stellen aan ADE. De enigste preventieve maatregelen die nu beschikbaar zijn richten zich op het elimineren van broedplaatsen van muggen, verbetering van bewustwording en kennis van knokkelkoorts in de algemene populatie over te nemen voorzorgsmaatregelen en de tekenen die wijzen op een gecompliceerd beloop zodat doorsturen van een patiënt naar het ziekenhuis aangewezen is.

**Hoofdstuk 11** onderzoekt of onderwijs en voorlichting de kennis over knokkelkoorts beïnvloedt in onze prospectieve dengue-studie. Onderwijs werd gegeven in de vorm van lezingen, posters en hand-outs waarin de tekenen en symptomen van dengue werden uitgelegd evenals de methoden van transmissie en hoe de ziekte voorkomen kan worden. Voor de interventie werd een mondelinge vragenlijst afgenomen door de studie artsen om deelnemers hun kennis met betrekking tot knokkelkoorts te kunnen vaststellen. De dezelfde vragenlijst werd 18 maanden later opnieuw afgenomen. Van de 2340 deelnemers die de beide tests voltooiden, scoorde slechts 0,3% van de deelnemers 'uitstekend' op de eerste test waarbij echter 8,4% 'uitstekend' op de tweede test scoorde. Ook het aantal deelnemers dat 'zeer slecht' scoorde verbeterde (1,4% versus 4,0%). De gemiddelde ruwe scores voor de pre en post tests waren respectievelijk 7,8 en 10.1. Verbetering van individuele scores correleerde sterk met het opleidingsniveau. Deze bevindingen tonen aan dat onze prospectieve studie leidde tot kennisverbetering bij vrijwilligers over van dengue.

**Hoofdstuk 12** beschrijft een studie waar families van 53 gehospitaliseerde knokkelkoorts patiënten en hun naaste buren in het westen van Jakarta gedurende twee weken werden gevolgd. In totaal zijn 785 familieleden of nabije buren geobserveerd en werden 192 (24,5%) knokkelkoorts infecties gedocumenteerd, bestaande uit 175 (22,3%) gevallen optredend vóór of tijdens de inclusie in de studie en 17 (2,2%) ontwikkelde knokkelkoorts tijdens de twee weken observatie periode. Van deze 17 nieuw ontwikkelde knokkelkoorts gevallen, hadden 8 personen geen symptomen, 8 koorts en was er één DHF grade II infectie. Deze studie geeft aan dat voor elke ziekenhuis patiënt met knokkelkoorts er ongeveer 3,6 andere knokkelkoorts infecties voorkomen in de nabijheid van de geïndexeerde patiënt. Deze studie toont verder aan dat

asymptomatische gevallen ook aanzienlijke bijdragen kunnen leveren aan de verspreiding van dengue virus; aangezien viremia ontdekt werd in twee van de acht.

# List of publications

## List of publications


1. Myint KS, Kosasih H, Artika IM, Perkasa A, Puspita M, et al. (2014) West Nile virus documented in Indonesia from acute febrile illness specimens. Am J Trop Med Hyg 90: 260-262.

2. Kosasih H, Bratasena A, Pangesti K, Laras K, Samaan G (2014) Managing Seasonal Influenza: Oseltamivir Treatment Policy in Indonesia? Acta Medica Indonesiana - The Indonesian Journal of Internal Medicine 46: 56-65.

3. Kosasih H, Roselinda, Nurhayati, Klimov A, Xiyan X, et al. (2013) Surveillance of influenza in Indonesia, 2003-2007. Influenza Other Respir Viruses 7: 312-320.

4. Kosasih H, de Mast Q, Widjaja S, Sudjana P, Antonjaya U, et al. (2013) Evidence for endemic chikungunya virus infections in bandung, indonesia. PLoS Negl Trop Dis 7: e2483.

5. Kosasih H, Alisjahbana B, Widjaja S, Nurhayati, de Mast Q, et al. (2013) The diagnostic and prognostic value of dengue non-structural 1 antigen detection in a hyper-endemic region in Indonesia. PLoS One 8: e80891.

6. Kosasih H, Widjaja S, Surya E, Hadiwijaya SH, Butarbutar DP, et al. (2012) Evaluation of two IgM rapid immunochromatographic tests during circulation of Asian lineage Chikungunya virus. Southeast Asian J Trop Med Public Health 43: 55-61.

7. Kosasih H, Ibrahim IN, Wicaksana R, Alisjahbana B, Hoo Y, et al. (2011) Evidence of human hantavirus infection and zoonotic investigation of hantavirus prevalence in rodents in western Java, Indonesia. Vector Borne Zoonotic Dis 11: 709-713.

8. Jeremijenko A, McLaws ML, Kosasih H (2007) A tsunami related tetanus epidemic in Aceh, Indonesia. Asia Pac J Public Health 19 Spec No: 40-44.

9. Suwandono A, Kosasih H, Nurhayati, Kusriastuti R, Harun S, et al. (2006) Four dengue virus serotypes found circulating during an outbreak of dengue

fever and dengue haemorrhagic fever in Jakarta, Indonesia, during 2004. Trans R Soc Trop Med Hyg 100: 855-862.

10. Kosasih H, Yusuf H, Sudjana P, Alisjahbana B, Wuryadi S, et al. (2006) Report of four volunteers with primary, secondary and tertiary dengue Infections during a prospective cohort study. 30.

11. Kandun IN, Wibisono H, Sedyaningsih ER, Yusharmen, Hadisoedarsuno W, et al. (2006) Three Indonesian clusters of H5N1 virus infection in 2005. N Engl J Med 355: 2186-2194.

12. Raekiansyah M, Pramesyanti A, Bela B, Kosasih H, Ma'roef CN, et al. (2005) Genetic variations and relationship among dengue virus type 3 strains isolated from patients with mild or severe form of dengue disease in Indonesia and Thailand. Southeast Asian J Trop Med Public Health 36: 1187-1197.

13. Porter KR, Beckett CG, Kosasih H, Tan RI, Alisjahbana B, et al. (2005) Epidemiology of dengue and dengue hemorrhagic fever in a cohort of adults living in Bandung, West Java, Indonesia. Am J Trop Med Hyg 72: 60-66.

14. Laras K, Sukri NC, Larasati RP, Bangs MJ, Kosim R, et al. (2005) Tracking the re-emergence of epidemic chikungunya virus in Indonesia. Trans R Soc Trop Med Hyg 99: 128-141.

15. Beckett CG, Kosasih H, Faisal I, Nurhayati, Tan R, et al. (2005) Early detection of dengue infections using cluster sampling around index cases. Am J Trop Med Hyg 72: 777-782.

16. Porter KR, Tan R, Istary Y, Suharyono W, Sutaryo, et al. (2004) A serological study of Chikungunya virus transmission in Yogyakarta, Indonesia: evidence for the first outbreak since 1982. Southeast Asian J Trop Med Public Health 35: 408-415.

17. Beckett CG, Kosasih H, Tan R, Widjaja S, Listianingsih E, et al. (2004) Enhancing knowledge and awareness of dengue during a prospective study of dengue fever. Southeast Asian J Trop Med Public Health 35: 614-617.

18. Beckett CG, Kosasih H, Ma'roef C, Listiyaningsih E, Elyazar IR, et al. (2004) Influenza surveillance in Indonesia: 1999-2003. Clin Infect Dis 39: 443-449.

# Acknowledgements

The book which is now in your hands is the outcome of a remarkable collaboration between devoted volunteers and dedicated researchers. Personally, I consider the introductions, meetings and opportunities of working together with these great people an exceptional gift.

For that reason, my first thank is to the volunteers who were willing to participate in the research we conducted, those who were willing to forgo time, provided their blood and other specimens to produce the data which become the contents of this thesis.

Dr Bachti Alisjahbana is one of a very few people whose hobby is to push people to pursue a doctoral degree. I am one of his "victims". I have long been urged by him to join this program. I have never took it seriously until the time came that I was asked to meet Prof Andre van der Ven in Bandung on one night in the year 2010. I was just nothing to lose as the word "research" has been erased from my mind since NAMRU-2 closed down. To my surprise, it turned out that that meeting became the beginning of my motivation. Prof Andre, along with Dr Bachti and Dr Quirijn de Mast welcomed me with open arms, constructed a plan for my education program, and searched for funding for all the costs. With patience they assisted me so that I am able to finish writing several manuscripts and this thesis. Without these three highly respected supervisor and co-supervisors, today we would not be holding this book and there would be no "Hora est" and ceremonies. Thus, I would like to convey my highest gratitude to my supervisor, Prof Andre for this once in a life time opportunity and his brilliant solution and research ideas when I was stuck. Also, a million thanks to my co-supervisors, Dr Bachti who always motivates me and tries hard to help despite his 24-hour busy schedules, and Dr Quirjn for the fruitful discussion to elaborate contents of the manuscript and his critical review.

Research in the medical field is not a popular profession or the goal of students and doctors as this field is considered 'boring' and 'dry'. That is why I am thankful to the many of those who introduced that "research can be fun and challenging." They are my former superiors at the Naval Medical Research Unit no 2, Jakarta, Indonesia (1998-2010): Dr Kevin R. Porter, Dr Charmagne G Beckett, Dr Patrick

J Blair, Dr Timothy H Burgess, and Dr Maya Williams. Thank you for working hard to find projects and grants so that we could conduct good research and enhanced the research capacities on sites. Also, I really appreciate that you shared the knowledge about virology, and trained us on how to write study proposals, protocols and manuscripts. To all my Indonesian colleagues, Ibu Ratna I Tan who was the first person asking me to find out about the PhD program in the Netherlands, Ibu Nami, Dr Erlin Listijaningsih, Ungke Antonjaya, Sri Hartati, and my other colleagues, those of the Viral Diseases program and the other departments, thank you very much for sharing the beautiful time together in the laboratories, and also in the badminton court (sneaking from work).

To the manuscript committee: Prof. dr. R. de Groot, Prof. dr. K van der Velden and Prof dr Ida Parwati, thank you very much for reading and approving this doctoral thesis.

To my four colleagues, Dr Susana Widjaja, dr Nurhayati, dr Susanto, and Haditya L Mukri, without whom would I be able to analyze the research data, moreover to finish this thesis. I am extremely thankful to you for all your help. It is now the time for the last three persons to pursue their S3 degree as well.

To the researchers whom I collaborated with from the beginning in developing hospital and community based study on hantavirus, dengue and chikungunya studies in Jakarta as well as in Bandung: the late drh Suharyono Wuryadi, dr Rita Kusriastuti, dr Agus Suwandono, Pak Djoko Yuwono, Pak Bambang Herianto, Pak Syahrial Harun, drh Ima Nurisa Ibrahim, dr Tatang K Samsi, dr Primal Sudjana, Dr Hadi Jusuf, Prof Ida Parwati, dr Ardini Raksanagara, dr "Fifi" Rudiman, Dr Rudi Wisaksana, dr Yumilia Hoo, dr Panji Hadisoemarto, dr Silvita, Ibu Hadijah, dr Beni, dr Kartika, dr Dewi Liana, dr Mieke, dr Safie, Nonon, Diana, Wulan, Evan Susandi, and also to all of the doctors in Indonesia who participated in the influenza surveillance, I thank you for your time, guidance and cooperation.

Avian influenza, is the beginning of an opportunity for me to return to the research world. I am very grateful for the trust shown by the late Dr Endang R Sedyaningsih, Prof David H Muljono, Pretty Multihartina, PhD, and Dr Frank

Mahoney from the US-CDC to ask me to be involved in the preparation for the establishment of WHO-CC. I also thank my colleagues from the Center of Biomedics and Health Basic Technology and US-CDC Jakarta for a close collaboration during this period, dr Tim Uyeki, my guru in writing articles on influenza, Gina Samaan for her support and interesting discussions, dr Khin Saw Aye Myint for the cooperation with Universitas Padjadjaran and Kendra Chittenden of the USAID for her interest in supporting scientific publications.

The network of Indonesia Research Partnership on Infectious Diseases (INA-RESPOND) became my next endeavor. In this opportunity, I would like to express my sincere gratitude to Dr Trihono, Dr Cliff Lane, Dr Siswanto, and Drs Ondri for their trust to have me on board, and to all members of the INA-RESPOND steering committee, Prof Emiliana Tjitra and dr Delima who have spent much of their time since the beginning of the establishment of this network to present. My special thanks to three most dedicated persons, Ibu Nikki Gettinger, Dr Sophia Siddiqui, and Dr M. Karyana for the flexibility and freedom I was given in terms of working hours so that I was able to finish my thesis during my tenure at INA-RESPOND. I really enjoy working with you. To my colleagues at the secretariat of INA-RESPOND, the Center of Applied Health Technology and Clinical Epidemiology, junior and senior researchers on sites, and Social and Scientific System, thank you for working so hard to make INA-RESPOND a reputable clinical research network in our region.

Room 456 will be the most memorable room in the Netherlands for Indonesian students. We even have a favorite spot that we call it Indonesian corner. Hence, I would like to thank those who shared this room and/or who often visited it; 1) Dr Reinot van Crevel, Dr Denise Telgt, Dr Monique Keuter, Dr Henri van Asten for their hospitality and guidance. 2) my friends in this "perguruan silat" : dr Rovina Ruslami, dr Achmad Rizal, dr Shelly Iskandar, who have completed their journey here; dr Agnes Ariantana, dr Erni Nelwan, Lidya Chaidir, Khutso Mothapo, dr Susan van Santen, Hinta Meijerink, dr Meta Michels, dr Ghozali whom I believe will follow very soon; 3). To those who have made my travel, stay, and work in the Netherlands enjoyable and legal: Mieke Daalderop, Maureen Libosan, and Carla IJsenbrandt. 3) The two special ladies: Lidya Chaidir and dr Rahajeng N Tunjungputri with whom I spent most of my time here, at the boarding room,

and downtown Nijmegen. 4). To Khutso Mothapo and dr Rahajeng, who have spent their valuable time to be the paranymphs this afternoon. Thank you all for the support, sharing sessions, and the dinners we had. Without all your friendship, my stay will not be as pleasant as it was.

I am very fortunate to have these amazing friends to whom I thank very much 1)Those who live in Netherlands: Chris who helped me settled in here for the first time, Dede, Richard, and Agus, to whom I go to out of boredom from the boarding room. 2). Those who kept nagging when I would finish my study: Ibu Wita, Kanti Laras, Saraswati, Gustiani Salim, Sukmawati, and others. Although annoying, it turns out to be of high motivation to me, to do my utmost (due to embarrassment). 3). To dr Aly Diana, Arditya Pradana and Fiona Mac Iver, who patiently helped in reading and editing this thesis. 4).Tobias and Chris who helped me prepare the first draft of the 'samenvatting' before being revised by Prof Andre.

Last but not least, the education I have pursued will not have been possible without the great sacrifice of my late parents. I am forever grateful to them. As in their minds physician has to practice and therefore, the profession I am now in is not what they thought a physician should be, I hope this thesis would make them quite happy. Also, the extraordinary support I received during my study, work and writing of this thesis was shown by my siblings, relatives and my close friends: Ida, Ridwan, Johannes, Nining, Heru, Jeffrey, dr Dewi Lokida, and dr Susana Widjaja. I am greatly thankful to them.

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Herman Kosasih was born in Bandung, Indonesia on 10th of September 1965. He graduated from the Faculty of Medicine, Universitas Padjadjaran Bandung in 1990. After working as a medical doctor at Wawo Utara, Bima, West Nusa Tenggara and Dieng Plateau, Banjarnegara, Central Java, he spent several years to elaborate "applied linguistics", another field that he was interested in at Atmajaya University, Jakarta. Since 1998, he worked at the Viral Diseases Program, US Naval Medical Research Unit (NAMRU)#2, Jakarta until 2010. During these 12 years, he participated in dengue, chikungunya, hantavirus and influenza studies.

In 2010-2011, he helped in the establishment of Indonesia as the WHO Collaborating Center on Influenza at the Human-Animal Interface. Since September 2010 until now, he works for a newly research collaboration between the National Institute of Health Research and Development (NIHRD) Ministry of Health, Republic of Indonesia, the US National Institute of Health, and medical faculties / referral hospitals throughout Indonesia, which is known as Indonesia Research Partnership on Infectious Disease (INA-RESPOND).